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**Dissecting the role of PML-II in gene transcription
and in IFN alpha-mediated apoptosis**

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. All the work recorded in this thesis is original unless otherwise acknowledged in the text or reference. None of the work has been submitted in any previous application for any degree. Parts of the data in Chapter 3 of this thesis have been published in Chen et al. (2015), a paper jointly authored by myself and others in which the elements included here were my own work.

Xueqiong Meng

Abbreviations

AKT	Protein kinase B
AP-1	Activator protein 1
APL	Acute promyelocytic leukemia
BAFs	BRG-1/BRM associated factors
Brg-1	Brahma-related gene 1
CBP	CREB-binding protein
CIITA	Class II transactivator
CML	Chronic myeloid leukemia
CNS	Central nervous system
cPML	cytoplasmic PML
CREB	cAMP-response-element binding protein
DAXX	Death-associated protein 6
dsRNA	Double-stranded RNA
DUBs	Deubiquitinases
DUSPs	Dual-specificity phosphatases
EC	Embryonal carcinoma
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
ESCs	Embryonic stem cells
FADD	Fas-associated death domain
FLIP	Flice inhibitory protein
FoxO3a	Forkhead box O3A
GAS	Gamma interferon activation site
GBP1	Guanylate-binding protein 1
H3K9me3	Histone H3 lysine 9 trimethylation
HAdVC-5/Ad5	Human adenovirus type 5
HAS	Helicase/SANT-associated
HATs	Histone acetyltransferases
hBRM	Human brahma
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylases

HIPK2	Homeodomain-interacting protein kinase-2
HMG	High mobility group
HMTase	Histone methyltransferases
HP1	Heterochromatin protein 1
HSV1	Herpes simplex virus type 1
IAP	Inhibitors of apoptosis
IFITM1/3	IFN α -induced transmembrane protein 1/3
IFN α /β/γ/λ	Interferon alpha/beta/gamma/lambda
IFNAR	IFN α /β receptors
IP3R	Inositol triphosphate receptor
IRF3/7/9	IFN regulatory factor 3/7/9
ISGs	IFN-stimulated genes
ISRE	Interferon-stimulated response element
JAK1	Janus kinase 1
KLHL2	Kelch like family member 2
MAMs	Mitochondria-associated membranes
MAPPs	Mitotic accumulation of PML proteins
MEFs	Mouse embryonic fibroblasts
MHC1	Major histocompatibility class 1
MLL1	Mixed-lineage leukemia 1
mTOR	Mammalian target of rapamycin
NES	Nuclear export signal
NF-κB	Nuclear factor-κB
NLS	Nuclear localization signal
OAS1	2'-5'-Oligoadenylate synthetase 1
p70S6K1	p70S6 kinase
PAMPs	Pathogen-associated molecular patterns
PI3K	Phosphatidyl-inositol (PI) 3'-kinase
PIC	Preinitiation complex
PKR	Protein kinase RNA-activated
PML	Promyelocytic leukaemia protein
PML-NBs	PML nuclear bodies
poly(I:C)	polyinosinic:polycytidylic acid

PP1A	Protein phosphatase 1A
PP2A	Protein phosphatase 2A
PRRs	PAMPs recognition receptor
PTMs	Post-translational modifications
PUMA	p53 upregulated modulator of apoptosis
QLQ	Glutamine-leucine-glutamine motif
RAR α	Retinoic acid receptor alpha
Rb	Retinoblastoma
RBCC	<u>R</u> ING-finger, two <u>B</u> -boxes (B1 and B2) and α -helical <u>C</u> oiled- <u>C</u> oil domain
RNA pol II	RNA polymerase II
SIM	SUMO-interacting motifs
STAT1	Signal transducer and activator of transcription 1
TFs	Transcription factors
TPR	Tetratricopeptide motifs
TRAIL/Apo2L	Tumour necrosis factor-related apoptosis-inducing ligand
TSSs	Transcription start sites
TYK2	Non-receptor tyrosine kinase 2
XAF-1	X-linked inhibitor of apoptosis (XIAP) associated factor-1
XIAP	X-linked inhibitor of apoptosis

Summary

Multiple isoforms of promyelocytic leukaemia protein (PML) participate in various cellular activities including innate immune responses, gene transcription and cell apoptosis. Recent work in our laboratory demonstrated PML isoform II (PML-II) was required for type I interferon (IFN) and IFN-stimulated gene (ISG) transcription by regulating transcription factor recruitment at gene enhancers/promoters. This thesis examines the mechanistic role of PML-II in this process.

Considering that the different C-terminal fragments of PML isoforms play distinct roles in PML function, the function of the PML-II C-terminal domain was investigated. A C-terminal region 615-758, defined by deletions $\Delta 1$ and $\Delta 2$, was essential for its function in the IFN response, while the N-terminal RBCC structure was dispensable. Removal of residues 615-758 greatly impaired PML-II binding with transcription factors NF- κ B and STAT1, coactivator (CBP) and SWI/SNF chromatin remodeling complex component Brg-1. These binding sites were further refined using smaller deletions to show that residues 645-665 were critical for PML-II binding with these transcription-related factors.

The effect of PML-II on chromatin remodeling and histone modification at target promoters was investigated. Both PML-II and Brg-1 regulated the recruitment of transcription factor (STAT1) and the enrichment/disposition of H3K9me3 histone marks at ISG promoters but had no effect on H3K4me3 at these promoters. Depletion of PML-II also impaired ISG promoter binding by SWI/SNF core subunits Brg-1 and BAF155. Recruitment of activators, coactivators, and other regulators/mediators to ISG promoters occurred sequentially and was interrupted by PML-II depletion. This suggested that PML-II is required for forming and stabilizing the whole transcriptional complex which contains various factors including STAT1, CBP, Brg-1 and BAF155.

Finally, the role of PML-II in IFN α -induced cell apoptosis was studied. Knockdown of PML-II enhanced ERK and AKT signaling, suggesting activation of both pro-survival pathways. Moreover, depletion of PML-II greatly decreased expression of IFN α -induced pro-apoptotic proteins including ISG15/54, OAS1, PUMA and TRAIL. It also impaired IFN α -mediated inhibition of AKT signaling and consequently increased cell resistance to IFN α -induced cell apoptosis.

Collectively, in this study we have shown the specific involvement of the PML-II C-terminal domain in IFN responses, and mapped the sequences within this domain that are necessary for its interaction with relevant transcription factors. I have also examined in detail the physical and functional interaction of PML-II with the SWI/SNF complex and shown the points in the transcription activation pathway at which PML-II acts. Finally, we have shown how impaired PML-II activity reduces the pro-apoptotic signaling that normally occurs downstream of an IFN response, suggesting a mechanism by which PML may provide its known tumour-suppressor function.

Chapter 1 Introduction

1.1 PML and isoforms

1.1.1 PML and PML-NB

Promyelocytic leukaemia protein (PML), was initially identified from patients with acute promyelocytic leukemia (APL) in which PML and retinoic acid receptor alpha (RAR α) genes form a fusion protein due to a t(15,17) chromosome translocation (de Thé et al, 1991; Goddard et al, 1991; Kakizuka et al, 1991). In normal cells, PML proteins form nuclear multiprotein complexes known as PML nuclear bodies (NBs). PML-NBs are heterogeneous and dynamic structures, which contain various components proteins such as SUMO-1 (Zhong et al, 2000a), Sp100, CREB-binding protein (CBP) (Doucas et al, 1999; LaMorte et al, 1998), Daxx (Zhong et al, 2000c), pRB and p53 (Fogal et al, 2000; Hofmann & Will, 2003). PML protein is the essential component of PML-NB. The formation of PML-NBs relies primarily on the self-association property of PML protein via its RBCC structure (Lang et al, 2010). PML SUMO binding motif and RING domain are necessary for PML-NB formation (Brand et al, 2010). The sumoylation of PML facilitates the recruitment of SUMOylated partner proteins and/or proteins containing binding motifs into the inner core of PML-NBs, which finally promotes the maturation of these spherical bodies (Brand et al, 2010; Lang et al, 2010; Shen et al, 2006). Therefore PML may function as the scaffold of this structure, allowing other proteins to shuttle in and out.

PML is known to have multiple isoforms due to mRNA alternative splicing, and the expression and function of those splicing variants is not yet fully understood (Condemine et al, 2006; Fagioli et al, 1992; Jensen et al, 2001). These PML isoforms and components move between NBs dynamically, thus suggesting that the PML-NB composition might be heterogeneous and functionally different (Weidtkamp-Peters et al, 2008). PML-NBs are dynamic macromolecular structures altering their number, size, and content in response to diverse stimuli such as viral infections, extracellular signals and genotoxic stress (Borden, 2002).

1.1.2 Isoforms

PML are classified into seven principle isoforms and designated PMLI-PMLVIIb (Jensen et al, 2001) (Figure 1.1). Each of PML I-V may also have several variants depending on the differential splicing pattern of exons 4-6 (Jensen et al, 2001). PML VII is a cytoplasmic variant of PML because it lacks exon 6 that contains the nuclear localization signal (NLS) and thus cannot move into nucleus (Reymond et al, 2001a). All PML isoforms include exons 1-3 and thus share an identical N-terminal region known as an RBCC motif that comprises a RING finger domain, two B-boxes, and a coiled-coil domain; they are distinguished by their unique C-terminal regions (Jensen et al, 2001). The RBCC domain is thought to play an important role in PML protein biological function (Jensen et al, 2001): it mediates a strong homo/hetero dimerization activity to interact with another identical partner protein (homo), or a distinct partner protein (hetero), which is essential for the assembly of PML-NBs (Geng et al, 2012)

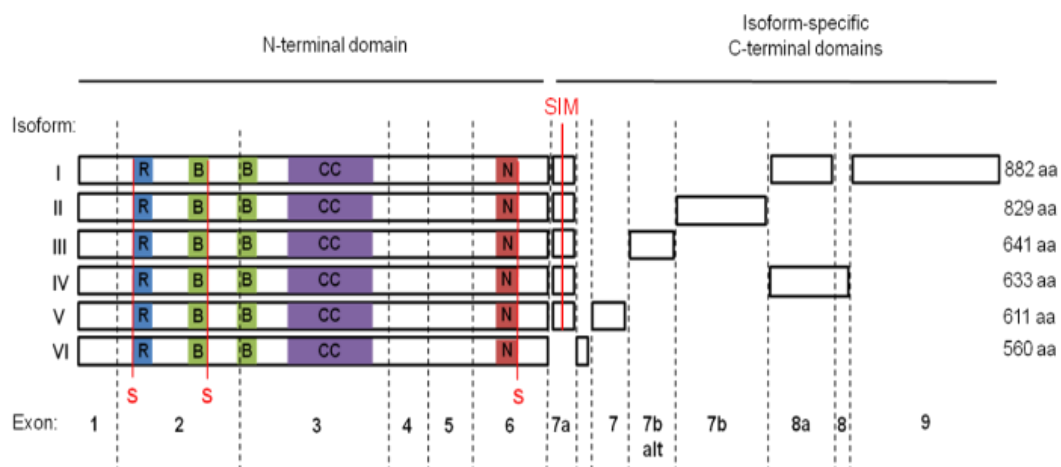


Figure 1.1 **Structure of PML protein isoforms.** All six nuclear PML isoforms contain three cysteine-rich zinc-binding domains: a RING-finger, two B-boxes (B1 and B2) and a predicted α -helical Coiled-Coil domain; three SUMOylation sites (S) and a nuclear localization signal (N). Only PMLI-V have SUMO-interacting motifs (SIM) (Wright, PhD thesis, University of Warwick, 2010)

1.1.3 PML protein C terminal domains

To date, several motifs have been identified in the C-termini of PML isoforms. The principal nuclear localization signal (NLS) is found at exon 6 among PMLI to PMLVI isoforms. However, a secondary NLS was demonstrated in the PML II C-terminus (Jul-Larsen et al, 2010). There is also a nuclear export signal (NES) (amino acids 704–713/exon 9), found only in PMLI, that is consistent with the partial nuclear and cytoplasmic distribution of this isoform. The SUMO-interacting motifs (SIM) is present only in PMLI to PMLV (Nisole et al, 2013).

Although PML-NBs assembly is always considered to depend on the RBCC motif in the N-terminal of all PML isoforms and a SUMO-SIM interaction network, the observation that PML-II and PML-V can form nuclear bodies and target to PML NBs independent of their N-terminal region indicated that the C-terminal domain of PML also contributed to assembly, maintenance, and structure stability of PML-NBs (Geng et al, 2012). Most recently, C-terminal motifs in PML isoforms have been demonstrated to have a critical role in the regulation of PML-NBs formation. The mutation of the SIM distinctively influences the structure of NBs formed by each individual PML isoform, with that of PMLI and PMLIV dramatically impaired. Several C-terminal elements that are also important in regulating NB structure, for example the 8b region in PML-IV, which function as a SIM thus possesses a strong ability to interact with SUMO-1 and SUMO-2, and critically participates in NB formation (Li et al, 2017).

It is becoming increasingly clear that different PML isoforms are functionally distinct (Bernardi & Pandolfi, 2007). The C-terminal of PML is thought to be associated with the specific biological properties of each isoform. Studies have shown that the difference between the C-terminal parts of PML isoforms is important for the recruitment of different interacting partners and therefore for the specific functions of each isoform (Geng et al, 2012).

1.1.4 Cytoplasmic PML (cPML)

Although PML is localized in the nucleus predominantly, there is a small portion of PML present in the cytoplasm (Carracedo et al, 2011; Giorgi et al, 2010; Lin et al, 2004). Alternative splicing is one of the major reasons for this localization. PML I to PML VI contains the NLS sequence in exon 6, leading to their nuclear localization. PML I contain NLS and NES which allows it shuttle between nucleus and cytoplasm. However, alternative splicing may produce isoforms lacking exon 6, thus resulting in their cytoplasmic retention. PML VIIb is an isoform that is devoid of NLS and localizes in the cytoplasm. However, PML VIIb is not the only isoform without NLS motif. Other PML isoforms identified include PML Ib and PML VIb are also found in cytoplasm (Lin et al, 2004; McNally et al, 2008; Raymond et al, 2001b). Essentially, all groups of PML isoforms may exist in three variants because of the possible omission of exons 4, 5 and/or 6. Among them, the variants lacking exon 6 are devoid of the NLS motif (Fagioli et al, 1992; Jensen et al, 2001). This suggests that there may be many alternative splicing cPML isoforms that distribute in the cytoplasm. In addition to the alternative splicing-generated cPML isoforms, cPML can also derive from the redistribution of nuclear PML. In the mitosis phase of the cell cycle, PML is redistributed from PML-NBs to the cytoplasm where it forms a specific complex termed mitotic accumulation of PML proteins (MAPPs) (Dellaire et al, 2006). The MAPPs may contribute to PML protein recycle to form new PML NBs in daughter nuclei. Moreover, viral infection may also redistribute nuclear PML to the cytoplasm (Borden et al, 1998; Kentsis et al, 2001).

Increasing evidence has demonstrated that cPML plays an important role in various cellular activities including antiviral responses, apoptosis, metabolism, tumorigenesis and cell cycle regulation (Jin et al, 2013). This finding has attracted lots of attention on the regulation and biological significance of cPML. However, how cPML regulates numerous cellular processes remains largely unknown. It is proposed that cPML may form multiprotein complexes similar to PML-NB, where it participate in various cellular processes. Consistent with this notion, the formation of cPML complexes with T β RI/T β RII/SARA/SMAD1/2 in early endosomes have been observed. This complex is required for inducing

TGF- β signaling activation (Lin 2004). Sequestration of cPML by TG-interacting factor (TGIF) dissociates this complex and thus negatively regulates TGF- β signaling (Seo et al, 2006). Inhibition of TGIF promotes complex formation and TGF- β signaling activation (Faresse et al, 2008). cPML was also observed to form a large complex in the endoplasmic reticulum (ER) and the mitochondria-associated membranes (MAMs) where cPML interacts with proteins such as phosphatase PP2A and kinase AKT and IP3 receptor regulates ER calcium release and apoptosis (Giorgi et al, 2010).

Recent studies revealed cPML interacts with the M2 isoform of pyruvate kinase (PKM2) and regulates its activity and glycolysis; this suggests an involvement of cPML in the regulation of cell metabolism (Shimada et al, 2008). However, due to the contrary effect of PKM2 on tumorigenesis, the exact functions of cPML on tumor suppression or tumor promotion remains unclear, this possibly is dependent on tumor types or distinct microenvironment (Cortés-Cros et al, 2013; Goldberg & Sharp, 2012). Although numerous biological functions have been attributed to cPML, little is known about how individual cPML isoforms are involved in distinct cellular processes, and the mechanism by which cPML itself is regulated remains poorly understood. It remains to be determined whether cPML is regulated by posttranslational modifications in a similar way to its counterpart in PML-NBs, which is regulated by modifications including SUMOylation, phosphorylation and ubiquitination (Carracedo et al, 2011; Cheng & Kao, 2012).

1.1.5 PML and post-translational modification

PML-NBs are a factory for protein modifications. It has been shown that PML regulates protein SUMOylation, phosphorylation, ubiquitination and acetylation (Carracedo et al, 2011; Guan & Kao, 2015). On the other hand, PML can also be extensively post-translationally modified itself by these same mechanisms (Cheng & Kao, 2012; Nichol et al, 2009). These modifications of PML add a complex layer of regulation to the physiological function of PML. Here, the

focus is on a brief review of the regulation function of PML on other protein modifications, on which there is very extensive literature.

PML-NBs host all kinds of enzymes capable of protein modification, such as acetyltransferases, deacetylases, ubiquitin and SUMO E3 ligases, deubiquitinases, phosphatases and kinases (Carracedo et al, 2011). The action of such enzymes creates a regulatory network that is of critical importance for cell homeostasis. Consequently, loss of PML results in deregulated modulation of protein function (Guan & Kao, 2015).

The predominant role of PML-NBs/PML in the regulation of protein acetylation has been widely demonstrated. For example, PML activates p53-dependent gene expression by promoting its acetylation by CBP in the PML-NBs (Pearson et al, 2000). The regulation of p53 by PML is a balance between the acetylation by CBP and the deacetylation by SIRT1, overexpression of which tilts the equilibrium towards p53 deacetylation and transcriptional repression (Langley et al, 2002). Interestingly, inactivation of PML, such as by formation of a PML-RAR α fusion protein, exerts the opposite effect on p53 activity, by promoting its deacetylation by a different family of HDACs deacetylases (Insinga et al, 2004).

Regulating the activity of E3-ligases and deubiquitinases (DUBs) is essential for PML protein stability and function. As an example, PML was found to inhibit KLHL2 activity by recruiting this enzyme to PML-NBs and this physically separates KLHL2 from its target (Lee et al, 2010). PML has also been shown to recruit MDM2 to a distinct subnuclear structure, the nucleolus, hence preventing p53 ubiquitination and consequent proteosomal degradation (Bernardi et al, 2004). Lastly, PML was found to modulate the activity of protein phosphatases, tuning the function of the protein phosphatases 1A and 2A (PP1A and PP2A). Loss of PML delocalizes PP1A and reduces its activity towards pRb, resulting in increased proliferation and reduced differentiation of neural progenitors (Regad et al, 2009). PML also positively regulates the activity of PP2A towards AKT in PML-NBs, and, as a result, losing PML leads to a more aggressive form of cancer (Trotman et al, 2006). The activity of several kinases to promote protein phosphorylation is also regulated by PML. For example, PML promotes the

phosphorylation of p53 by homeodomain-interacting protein kinase-2 (HIPK2) kinase, which increases the rate of acetylation of p53 and consequently its transcriptional activation (Hofmann et al, 2002). Together, many of the numerous functions attributed to PML are the result of its regulation of post-translational modification of target proteins.

1.2 PML tumor suppressor function and apoptosis

Numerous studies have shown that PML and PML NBs actively participate in many aspects of cellular processes such as oncogenesis, DNA damage responses and subsequent repair processes, stress responses (Lallemant-Breitenbach et al, 2001), apoptosis (Giorgi et al, 2010), senescence (Bischof et al, 2002), antiviral activity (Everett & Chelbi-Alix, 2007), interferon responses (El Bougrini et al, 2011) and transcription regulation (Chen et al, 2015).

The tumour suppressive function of PML was first suggested by the identification of PML-RAR α fusion protein in APL (de Thé et al, 1990; de Thé et al, 1991). Soon after, the tumour suppressive activity of PML was extrapolated to various solid tumours. Increasing number of studies have revealed that many cellular activities, such as growth inhibition, apoptosis, senescence, suppression of oncogenic transformation, and inhibition of tumour cell migration and angiogenesis, are related to PML (Chen et al, 2012). The inactivation or downregulation of PML would provide an advantage for tumour development and progression. Indeed, the expression level of PML protein is frequently reduced in diverse types of human tumours and this downregulation often correlates with tumour progression (Gurrieri et al, 2004; Trotman et al, 2006). Many lines of evidence indicate a role for PML in modulating apoptosis. For example, lymphocytes, thymocytes and embryonic fibroblasts derived from PML-null mice are more resistant to apoptosis induced by stimuli that activate either intrinsic or extrinsic apoptotic pathways when compared with their wild type counterparts (Guo et al, 2000a; Wang et al, 1998).

PML mediates pro-apoptotic functions by activating p53 through multiple mechanisms including by promoting p53 phosphorylation and acetylation by

recruiting it into PML-NBs and by binding and inhibiting the p53 negative regulator MDM2 (Bernardi & Pandolfi, 2003; Takahashi et al, 2004). Besides p53, several other factors such as Daxx which are implicated in the pro-apoptotic function of PML are recruited to PML-NBs and interact with sumoylated PML (Lin et al, 2006). Daxx is reported to repress the expression of several anti-apoptotic genes when localized in PML-NBs, thereby eliciting pro-apoptotic functions (Croxtton et al, 2006). PML also regulates expression of IFN α -induced pro-apoptotic protein TRAIL in hepatocellular carcinoma cells (Chawla-Sarkar et al, 2001) while the inhibition effect of IFN α on myeloma cell growth correlated with PML induction (Crowder et al, 2005). Cytoplasmic PML was also found to exert proapoptotic activity through the regulation of a formation of a large complex, involving PP2A, AKT, and the inositol triphosphate receptor (IP3R), in mitochondria-associated membranes (MAMs). The MAMs connect the endoplasmic reticulum (ER) and mitochondria, and play a central role in apoptosis through the regulation of calcium influx from the ER to the mitochondria. PML-deficient cells exhibited impaired calcium influx to the mitochondria and resistance to ER-stress-induced cell death (Giorgi et al, 2010; Pinton et al, 2011).

1.3 PML function in gene transcription

PML-NBs host a huge number of functional proteins. Many of these proteins can be physically and/or functionally linked to PML protein. Strikingly, many of these proteins are involved in transcriptional regulation, for example, transcriptional factors IRF3, NF- κ B, STATs (Chen et al, 2015), AP-1 and p53 (Guo et al, 2000a), coactivator CREB-binding protein (CBP) (Doucas et al, 1999), transactivator C/EBP β and corepressors HDAC and N-CoR (Shiio et al, 2006; Zhong et al, 2000b). Many transcription factors transiently associate with PML and PML-NB, in which their activity is modulated by posttranslational modification. This may result in either activation or repression of specific genes.

Also supporting a role for PML-NBs in transcription, they have been shown to move to localize adjacent to chromatin regions when they become

transcriptionally active, for example Major Histocompatibility Class 1 (MHC1) (Kumar et al, 2007; Shiels et al, 2001) and p53 loci (Shiels et al, 2001; Sun et al, 2003). Furthermore, PML colocalizes and binds with the histone acetyltransferase CBP, and colocalizes with RNA Pol II in a cell cycle-dependent manner (Chen et al, 2015; Kiesslich et al, 2002). It was reported recently that PML-II also interacts with Ad E1A-13S, and thus enhances E1A-mediated transcriptional activation (Berscheminski et al, 2013).

On the other hand, PML can repress transcription through its interaction with histone deacetylases and heterochromatin protein 1 (HP1) (Seeler et al, 1998; Wu et al, 2001) and through the regulation of heterochromatin recondensation in satellite DNA (Luciani et al, 2006). The transcriptional regulation and heterochromatin remodeling induced by PML have highlighted the importance of this tumour suppressor in the induction of cellular senescence (Bischof et al, 2002; Luciani et al, 2006; Ye et al, 2007), with recent evidence pointing at a role for PML in regulating this process through the Rb-E2F pathway (Vernier et al, 2011). Together, the fact that PML associates with a wide variety of transcriptional activators, transcriptional repressors and histone modifiers suggests an important role of PML in gene transcription.

1.4 Interferon (IFN)

In 1957, interferon was discovered as a secreted factor responsible for inhibition of viral growth (Isaacs & Lindenmann, 1957). Since then a large number of IFNs have been identified. Generally, IFNs can be classified into three main types, type I, type II and type III IFNs. The type I IFN family consists of multiple subtypes including 12 IFN α subtypes, IFN β , IFN ϵ , IFN κ and IFN ω (Pestka et al, 2004). By contrast, the type II IFN family only has only one member, IFN γ , a cytokine. The third type of IFNs is composed of IFN λ 1 (also known as IL-29), IFN λ 2 (also known as IL-28A) and IFN λ 3 (also known as IL-28B) (Sheppard et al, 2003). All three IFN types possess strong antiviral and immunomodulatory properties. In this thesis, the focus is mainly on type I IFN.

Type I IFNs can be produced by almost every cell type, including leukocytes, fibroblasts and endothelial cells. The induction of type I IFNs differs depending on the stimulus and the responding cell type. Type I IFN is often considered to be induced specifically by viruses, but actually they can also be induced in response to various pathogen-associated molecular patterns (PAMP)s such as viral glycoproteins, viral RNA, bacterial endotoxin (lipopolysaccharide), bacterial flagella and DNA CpG motifs (Randall & Goodbourn, 2008; Trinchieri, 2010). These PAMPs are recognized by different PAMPs recognized receptor (PRR)s, leading to the activation of transcription factors including IFN regulatory factor 3 (IRF3), IRF7 and nuclear factor- κ B (NF- κ B) (Akira et al, 2006; Kawai & Akira, 2008; Yoneyama & Fujita, 2009). The activated TFs, together with coactivator CBP, form a transcriptional complex (enhancersome) that binds to the *IFNB* promoter/enhancer to initiate the transcription of IFN β genes (Kim et al, 1998; Merika et al, 1998) (Figure 1.2).

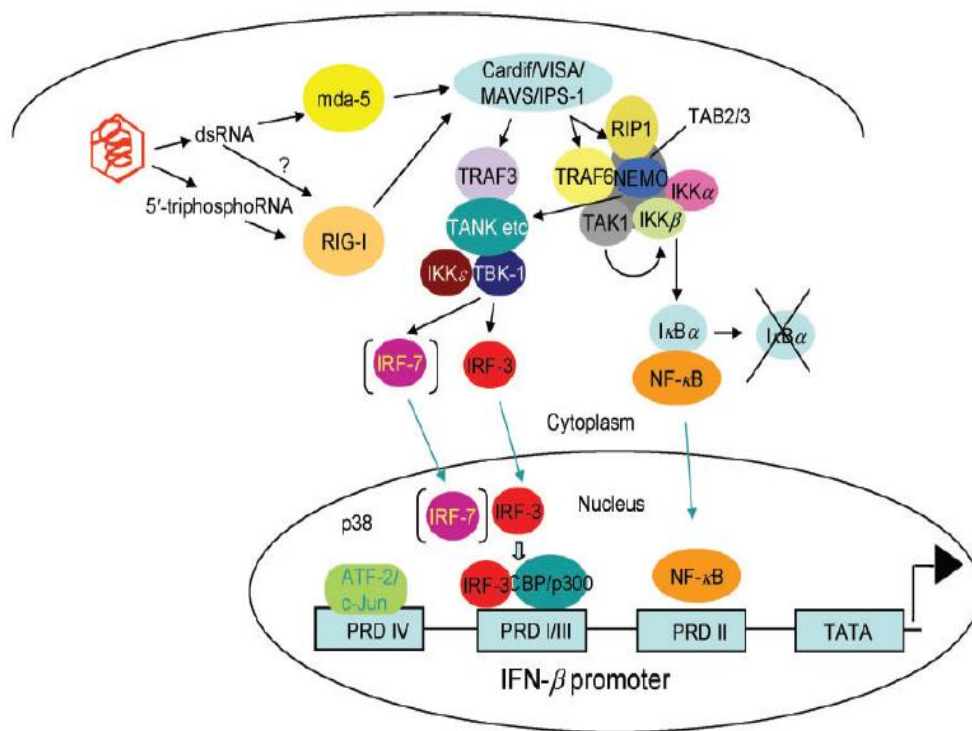


Figure 1.2 Signaling pathway of IFN β induction
(Randall & Goodbourn, 2008)

1.5 Type I IFN-mediated JAK-STAT signaling pathway

Following secretion from cells, type 1 IFNs mediate their effects by binding to heterodimeric cell surface receptors known as the IFN α/β receptors, IFNAR1 and IFNAR2. During IFN α or IFN β stimulation, IFNAR become associated with and activate Janus kinase 1 (JAK1) and non-receptor tyrosine kinase 2 (TYK2) (Darnell et al, 1994). Activated JAK1 and Tyk2 are auto-phosphorylated on tyrosine residues and this tyrosine phosphorylation activates the cytoplasmic latent STAT1 and STAT2. Activated STATs form STAT1/STAT2 heterodimers complexes that translocate to the nucleus, where they bind with IRF9 (p48) to form the interferon-stimulated gene factor-3 (ISGF3), STAT1-STAT2-IRF9 trimeric complex. The resulting ISGF3 complex recognizes and binds to the specific interferon-stimulated response element (ISRE) sequence in the promoter of interferon-stimulated genes (ISGs) (Figure 1.3) (Darnell, 1997; Darnell et al, 1994). ISGs products regulated by type 1 IFNs are the primary effectors of IFN-mediated biological functions.

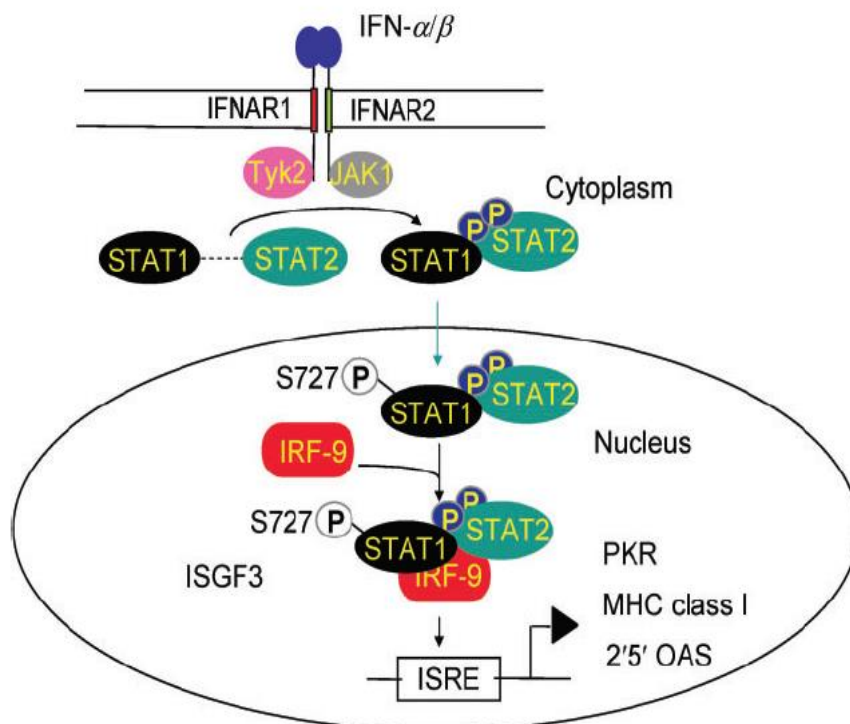


Figure 1.3 IFN α/β activated JAK-STAT signaling pathway
(Randall & Goodbourn, 2008)

1.6 IFN-stimulated genes

1.6.1 ISG56

IFN-stimulated gene 56 (ISG56), also known as IFIT1, is the defining member of the ISG56 gene family. ISG56 is not expressed in most untreated cells, however viral infection or type I IFN stimulates rapid induction of the ISG56 gene. Like its homologue, ISG54, ISG56 contains multiple tetratricopeptide (TPR) motifs and has two ISRE in its promoter so its transcription can be activated by ISGF3; it is also directly activated by IRF3. ISG56 is an important suppressor of viral replication, and is reported to be active against hepatitis C virus, West Nile virus, and lymphocytic choriomeningitis virus (Wacher et al, 2007; Wang et al, 2003). The C-terminal of ISG56 is essential for an interaction with the translation factor eIF-3e, which inhibits eIF-3 induced translation initiation and hence affects protein synthesis (Guo et al, 2000b; Hui et al, 2003). A recent study revealed virus-induced ISG56 also negatively regulated the activation of IRF3 and NF- κ B through a specific association with stimulator of interferon genes (STING/also known as MITA) that disrupted the interaction of STING/MITA with VISA or TBK1; ISG56 thus also plays a negative-feedback role in the IFN antiviral response (Li et al, 2009).

1.6.2 ISG54

The ISG54 gene, also known as IFIT2, encodes a protein of 54 kDa (Reich, 2013). ISG54 protein is another member of the ISG56/IFIT1 family which is comprised of ISG54, ISG56/IFIT1, ISG58/IFIT5, and ISG60/IFIT3 in humans (Fensterl & Sen, 2011). ISG54 can be induced by type I and III IFNs, virus infection, and by molecular patterns such as double-stranded RNA or lipopolysaccharide. ISG54 gene has two ISRE elements in its promoter which can be recognized and bound by the ISGF3 under various stimuli, thus activating its transcription (Reich, 2013). Like ISG56, the activation of IRF3 also directly activates ISG54 expression: HCMV-induced activation of ISG54 has been reported to be mediated by a transcriptional complex that contains IRF3 (Grandvaux et al, 2002; Navarro et al, 1998). In addition to its function as

antivirus protein, ISG54 is also reported to work as a mediator of apoptosis and to promote cell apoptosis via a mitochondrial pathway (Reich, 2013; Stawowczyk et al, 2011). ISG54 normally does not exist as a monomer, but instead is present as ISG54/ISG54 homodimers and/or as heterodimers with ISG56 or ISG60. The binding partner of ISG54 affects ISG54 apoptosis function: for example, co-expression of ISG60 with ISG54 inhibits the effect of ISG54 on cell apoptosis (Stawowczyk et al, 2011).

1.7 PML function in IFN response

Several studies have demonstrated the functions of PML protein and PML-NBs in innate immunity (Geoffroy & Chelbi-Alix, 2011). For example, ectopic over-expression of PML inhibits the growth of IFN-sensitive viruses, influenza A virus replication is significantly repressed by over-expression of some PML isoforms, and correspondingly, depletion of PML leads to an enhancement in viral propagation (Iki et al, 2005; Tavalai & Stamminger, 2008). In addition, various viruses encode proteins that disrupt PML and/or PML-NB, including E4 Orf3 of human adenovirus type 5 (HAdVC-5, Ad5) (Ullman & Hearing, 2008; Ullman et al, 2007) and ICP0 of herpes simplex virus type 1 (HSV1) (Leib et al, 1999; Mossman et al, 2000) and viruses lacking these functions are unable to overcome IFN responses.

Within the type 1 IFN response, PML-IV was reported to regulate the cellular distribution of Pin1 (peptidyl-prolyl cis/transisomerase) and to prevent IRF3 degradation thus enhancing the synthesis of IFN β in response to virus infection (El Asmi et al, 2014). Recent work in our laboratory demonstrated that PML-II positively regulates IFN β transcription and IFN-mediated response. Knockdown of PML-II substantially inhibits the expression of IFN β and IFN-stimulated genes (ISGs) such as ISG15, ISG20, ISG54, ISG56, IRF7, PKR and IP-10 (Chen et al, 2015).

PML protein has also been shown to serve as a regulator in IFN γ signaling, by affecting STAT1 DNA binding and transcription activity (Choi et al, 2006; El

Bougrini et al, 2011). In addition, the presence of PML was found to enhance IFN γ -induced MHC Class II expression: PML-II in particular bound to the key transcription factor CIITA and stabilized it, causing it and the MHCII gene loci to associate with PML-NBs (Ulbricht et al, 2012).

1.8 Type I IFN anti-tumour function

The most important function of type I IFNs was initially considered to be an antiviral immune response, however, increasing evidence has shown that these cytokines are also involved in other cellular activities including anti-proliferative and pro-apoptotic effect and immune modulatory properties (Figure 1.4).

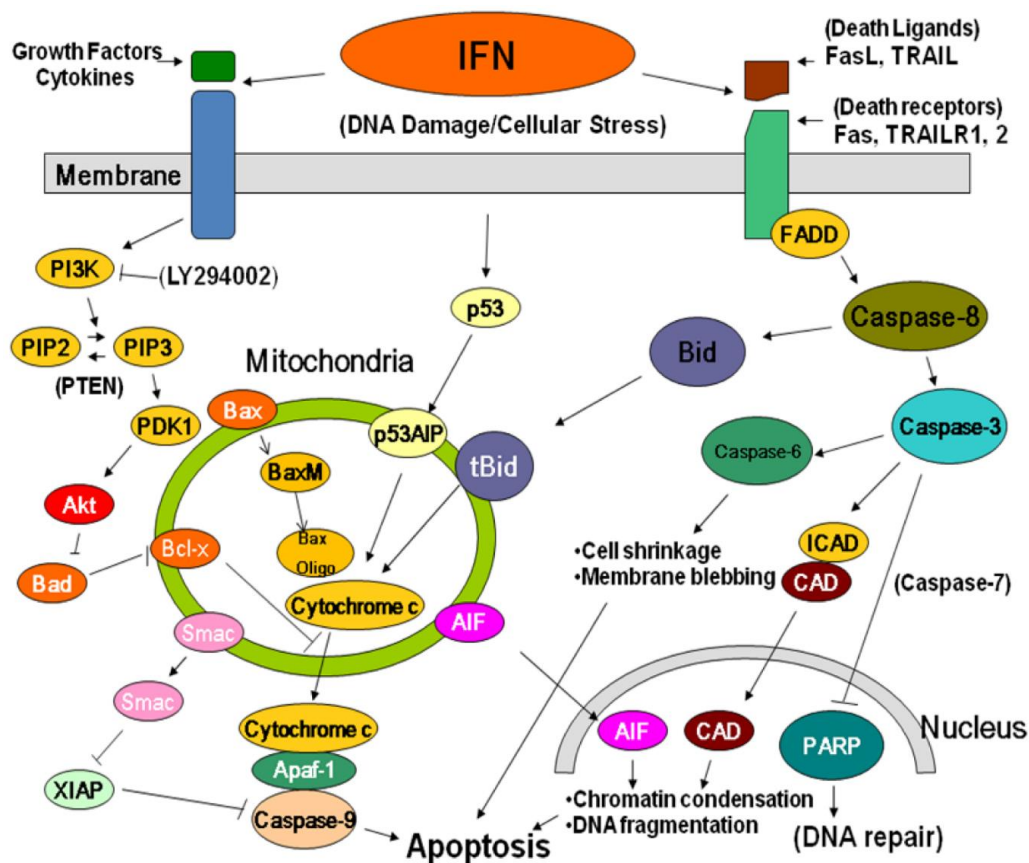


Figure 1.4 IFN-activated intrinsic and extrinsic apoptosis mechanisms
(Bekisz et al, 2010)

IFNs were the first human proteins to be effective in cancer therapy and were among the first recombinant DNA products to be used clinically. Type I IFNs

have been extensively used for the treatment of several types of cancer, including haematological malignancies (for example, hairy cell leukaemia and some B or T cell lymphomas) and solid tumours (for example, melanoma, renal cell carcinoma and Kaposi's sarcoma) (Ferrantini et al, 2007; Rizza et al, 2010). There are some studies suggesting that type I IFNs could act as an adjuvant in antitumour cancer vaccines (Di Pucchio et al, 2006; González-Navajas et al, 2012; Kirou et al, 2005). IFN α has also been used as a chemotherapeutic agent in combination with other anti-cancer drugs against hepatocellular carcinoma (HCC), and these clinical trials have demonstrated the considerable effectiveness of IFN in patients with HCC (Kaneko et al, 2002; Leung et al, 2002; Shaaban et al, 2014).

1.9 IFN α -regulated ERK signaling pathway

Regulating other (non-JAK-STATs) signaling pathways is also important for type I IFNs to mediate their pleiotropic biological effects (Inamura et al, 2005; Stancato et al, 1997; Uddin et al, 1997). For example, IFN α stimulation transiently activated the extracellular signal-regulated kinase 1 (ERK1) or ERK2 signaling pathway (Arora et al, 1999). The activated ERK pathway participates in the response of IFN to viral infection. ERK2 was reported to regulate IFN activation of early response ISGs by modifying the JAK/STAT signaling cascade (Arthur & Ley, 2013; González-Navajas et al, 2012). Moreover, type I IFNs also stimulated ERK and Raf-1 enzyme activity in a RAS-independent pattern (David et al, 1995; Stancato et al, 1997).

However, some controversial results have also reported that IFN α treatment reduces the phosphorylation of MEK1 and ERK1/2 in transformed human hepatocellular carcinoma (HCC) cell lines and hence reduces rather than increases ERK signaling. IFN α diminished phosphorylation of ERK after 24-48 hours stimulation and this was paralleled by reduced enzymatic activity and but this was independent of upstream RAS/and RAF-1 activation (Inamura et al, 2005; Romero et al, 2000). Also, knockdown of STAT1 or JAK1 suppressed the reduction of phosphorylation both of ERK and MEK and thus diminished the growth inhibition by IFN α . These results suggest that IFN α induces anti-

proliferative signaling via the JAK/STAT pathway and may reduce growth stimulation signaling by cross-talk with the MEK/ERK pathway.

Taken together, the data are clear that type I IFNs can regulate multiple signaling pathways, activating or suppressing them in different target cells, and that this regulation is essential for IFNs wide spectrum of biological activities such as antiviral activity, immunomodulatory and growth inhibitory effects.

1.10 IFN α -regulated PI3K/AKT signaling pathway

In addition to its effects on Jak/STATs and ERK signaling, type I IFN can also induce the activation of the serine kinase phosphatidyl-inositol (PI) 3'-kinase (PI3K)/AKT/mTOR signaling pathway in a STAT-independent manner (Kaur et al, 2008a; Kaur et al, 2008b; Uddin et al, 1997). Activation of the PI3K signaling cascade controls the activation of mammalian target of rapamycin (mTOR), regulating downstream p70S6 kinase (p70S6K1) activation and the subsequent phosphorylation of the S6 ribosomal protein, which in turn regulates mRNA translation (Kaur et al, 2008a; Lekmine et al, 2004; Lekmine et al, 2003). However, PI3K/AKT pathway activation does not play a role in the activation of the ISGF3 complex and transcriptional regulation of genes that contain ISREs in their promoters (Uddin et al, 1997). AKT activation is also unrelated to regulatory activities on IFN-dependent STATs phosphorylation/activation and had no effect on gene transcription (Lekmine et al, 2004; Lekmine et al, 2003). The activation of this signaling pathway by IFN may complement the function of IFN-activated JAK-STAT pathways, by allowing increased translation of ISG mRNAs and, ultimately, increased induction of the biological effects of IFNs. In terms of biological relevance, the activation of PI3K/AKT/mTOR signaling has been shown to mediate the antiviral effects of IFN α against the hepatitis C virus (Matsumoto et al, 2009). It is important to note that PI3K activation events reportedly occur very early (30min) in the cells after stimulation with IFN α and regulate the induction of early response ISGs such as ISG15, CXCL10 and/or IRF7 to mediate IFN antiviral responses, the biological significance of this effect remains unclear (Kaur et al, 2008a; Uddin et al, 1997). In contrast to the

activation of AKT signal by IFN α , a recent report showed AKT and NF- κ B signaling were inhibited by IFN β treatment in human cervical cancer cells, thus decreasing the expression of anti-apoptotic protein Bcl-2 (Ethiraj et al, 2016).

The function of the IFN-ERK/AKT axis in virus-infected cells and cancer cells is different. This is probably because, when cells require an immediate anti-virus function with corresponding short-term IFN release, IFN α may activate ERK/AKT signaling, while when cells are exposed constitutively to IFN α stimulation, the effect on ERK/AKT signaling is inhibitory, thus inhibiting cell proliferation and promoting cell apoptosis. The differing outcomes of IFN stimulation on ERK/AKT signaling may also suggest an IFN concentration-saturation threshold: when IFN concentration is saturating, it more likely to exert a pro-apoptosis function thus ERK/AKT signaling is inhibited, while when IFN is at non-saturating concentrations, IFN α may conversely activate ERK/AKT signaling and thus promotes cell survival.

1.11 IFN α -induced pro-apoptotic proteins

The expression products of pro-apoptotic ISGs are thought to be the primary effectors that mediate the IFN α antitumor function. A group of ISGs have been identified that are related to this biological response (Chawla-Sarkar et al, 2003; de Veer et al, 2001). For example, the increased expression of classical ISG15 (Zhou et al, 2017), ISG54 and OAS1 (2'-5'-Oligoadenylate synthetase 1) (Reich, 2013; Stawowczyk et al, 2011) and XAF-1 (XIAP associated factor-1), CD95 (Fas/APO-1), OAS1, PML and RIDs (regulators of IFN induced death) had been reported as one means by which IFN α induces cell apoptosis (Chawla-Sarkar et al, 2003). The TRAIL/Apo2L (tumor necrosis factor-related apoptosis-inducing ligand) was also demonstrated to be an important effector required for IFN α -mediated growth inhibition and apoptosis in myeloma and hepatocellular carcinoma (Crowder et al, 2005; Herzer et al, 2009). Moreover, the expression level of PUMA, a p53-dependent gene, was also increased in response to IFN α stimulation in human myeloma cells suggesting a possibility of PUMA being related to IFN α -induced apoptosis (Gómez-Benito et al, 2007). In addition to

inducing the expression of pro-apoptotic proteins, IFNs also directly induces caspase-4 and caspase-8 (Chawla-Sarkar et al, 2003; de Veer et al, 2001). Activated caspase-8 cleaves Bid, a pro-apoptotic member of Bcl2 family, resulting in disruption of mitochondrial potential and intrinsic apoptotic pathway activation.

1.11.1 Fas/CD95 and XAF-1

IFN α has been shown to induce apoptosis by inducing Fas/CD95 expression in multiple myeloma cells and leukemia cell lines (Selleri et al, 1997; Spets et al, 1998). Upregulation of Fas expression, induction of apoptosis and tumor regression were observed following injection of IFN α into basal cell carcinomas (Buechner et al, 1997). Like other TNF family members, Fas ligation resulted in FADD (Fas-associated death domain) activation, thus activating the extrinsic apoptosis pathway. However, the induction of Fas alone may not be sufficient to cause apoptosis as *in vitro* most tumor cells were resistant to Fas. However IFN- γ treatment sensitized melanoma and cholangiocarcinoma cells to Fas-induced apoptosis *in vitro* (Ahn et al, 2002; Ugurel et al, 1999).

XAF-1 (X-linked inhibitor of apoptosis (XIAP) associated factor-1), a candidate tumour suppressor ISG gene was identified ubiquitously in all normal adult and fetal tissues but was present in very low levels in a variety of cancer cell lines (Fong et al, 2000). Over expression of XAF-1 results in neutralization of the ability of XIAP to inhibit cell death (Liston et al, 2001). XIAP is one of the six known human inhibitors of apoptosis (IAP), it being shown that it bound directly to and inhibited caspase-3, caspase-9 and caspase-7 function (Deveraux et al, 1998; Deveraux et al, 1997). XIAP levels were high in the majority of cell lines. Both IFN α and IFN β effectively induced XAF-1 transcript in all cells examined however, induction of XAF-1 protein expression was observed only in cell lines sensitive to apoptotic effects of IFNs. IFN β may therefore inhibit XIAP function (and hence promote apoptosis) by induction of XAF-1. Although constitutive expression of XAF-1 in A375 melanoma cells did not induce apoptosis, it did render them sensitive to TRAIL-induced apoptosis (Leaman et al, 2002).

1.11.2 TRAIL

The TRAIL/Apo2L (tumor necrosis factor-related apoptosis (TNF)-inducing ligand), is a transmembrane protein that shares homology in its extracellular domains with other members of the TNF family (Hymowitz et al, 2000). Both *in vitro* and *in vivo* studies have demonstrated that TRAIL is without significant toxicity towards normal cells or tissues (Pitti et al, 1996). However, once TRAIL was bound to its receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), it resulted in activation of caspase-8 (pro-apoptotic) pathways.

TRAIL has been identified as an ISG (Chawla-Sarkar et al, 2001; Chen et al, 2001; Kayagaki et al, 1999) and has also been demonstrated to be an important effector required for IFN α -mediated growth inhibition and apoptosis in myeloma and hepatocellular carcinoma (Crowder et al, 2005; Herzer et al, 2009). In non-hematopoietic cells, such as melanomas and ovarian carcinoma, IFN β preferentially induced TRAIL in comparison to IFN α (IFN- α 2) and resulted in induction of apoptosis (Chawla-Sarkar et al, 2001; Morrison et al, 2001); in comparison, melanoma cells were resistant to IFN- α 2 and showed poor induction of TRAIL. However in multiple myeloma, IFN- α 2 can effectively induces TRAIL (Chawla-Sarkar et al, 2001; Morrison et al, 2001). The inhibitory effect of IFN α on myeloma cell growth correlated with PML and TRAIL induction (Crowder et al, 2005). The expression of IFN α -induced TRAIL was also greatly decreased by the loss of PML in hepatocellular carcinoma cells (Chawla-Sarkar et al, 2001) whilst RNAi silencing of PML downregulated TRAIL expression in hepatoma cells and correspondingly blocked IFN α -induced apoptosis (Herzer et al, 2009).

All these results suggest that TRAIL is essential in IFN α -induced growth inhibition or apoptosis and also suggest that PML has an important regulation function in IFN α -induced TRAIL expression.

1.11.3 PUMA

PUMA is a member of the Bcl-2 homology 3 (BH3)-only Bcl-2 family of proteins. The human *PUMA* gene contains three coding exons (exons 2–4) and two noncoding exons (exons 1a and b) (Nakano & Vousden, 2001). PUMA has multiple variants due to mRNA alternative splicing (Nakano & Vousden, 2001). PUMA α and PUMA β are two identified forms of encoded protein which have the BH3 domain (Nakano & Vousden, 2001). A BH3 domain is important for PUMA interacting with Bcl-2-like proteins such as Bcl-2 and Bcl-XL (Nakano & Vousden, 2001; Yu et al, 2001), while the C-terminal hydrophobic domain of PUMA directs its mitochondrial localization (Yee & Vousden, 2008; Yu et al, 2003). The *PUMA* gene contains several potential binding sites for the binding with various transcription factors such as p53 (Kaeser & Iggo, 2002), c-Myc (Fernandez et al, 2003) and forkhead box O3A (FoxO3a) (You et al, 2006). These transcription factors are rapidly recruited to their respective sites in the PUMA promoter and activate transcription by different stimuli.

Normally, the expression level of PUMA is very low in unstressed cells (Yu et al, 2001), but it can be rapidly induced by various stimuli including DNA damage, IFN α stimulation or virus infection (Gómez-Benito et al, 2007). Structure analysis showed that the PUMA promoter, exon 1a and intron 1 are enriched with a high ratio of guanine and cytosine nucleotides, which suggests secondary structures can be formed to inhibit transcription, and this may also account for the low basal levels of PUMA in unstressed cells (Ming et al, 2008; Yu et al, 2001). In contrast to other BH3-only proteins, activities of which are controlled by multiple mechanisms including posttranslational modification, PUMA activity is primarily transcription-dependent. PUMA induces apoptosis by interaction with anti-apoptotic Bcl-2 family members, thereby relieving their inhibitory effects on the pro-apoptotic proteins Bax and/or Bak, which finally results in mitochondrial dysfunction and caspase activation (Nakano & Vousden, 2001; Yu et al, 2001).

1.12 Eukaryotic genomes and gene expression

Eukaryotic genomes are packaged into chromatin which is a complex of macromolecules found in cells, consisting of DNA, protein and RNA. The primary protein components of chromatin are histones that compact the DNA. Gene transcription/expression can be initiated/activated by various external signals. In eukaryotic cells, gene transcription is complicated and strictly regulated process, which involves many factors and cofactors and their sequentially or ordered recruitment and assembly at gene promoter/enhancer sequences (Agalioti et al, 2000). This process has to occur, for example, during activation of genes such as IFN β and ISGs. In generally, transcriptional activation/initiation involves four key steps: (1) Binding specific transcription factors to enhancers and/or regulatory DNA sequences; (2) opening the chromatin by chromatin remodeling complex and histone modifiers; (3) Binding of mediator complex; (4) assembly of pre initiation complex (Wilson & Roberts, 2011).

1.13 Histone acetylation

The nucleosome is the basic unit of eukaryotic chromatin, consisting of 146bp of DNA wrapped around two molecules each of H2A, H2B, H3 and H4 (known as core histones). Histones H3 and H4 have long N-terminal tails which are often subjected to a variety of post-translational modifications (PTMs) such as acetylation, methylation, phosphorylation, ubiquitination and sumoylation (Li et al, 2007). Such modifications within particular sites/promoters create a global chromatin environment which affects the association of different factors or enzymes with those sites and eventually regulates the expression of the gene.

Histone acetylation is the best-studied histone modification involved transcription activation. Typically, transcriptionally active genes possess hyperacetylated histones around their promoters, while silent genes have hypoacetylated histones. HAT proteins p300/CBP, which were originally identified as proteins that bound to the adenoviral E1A and the cAMP-response-

element binding protein (CREB), respectively (Chrivia et al, 1993; Eckner et al, 1994), are essential for histone acetylation. Acetylation of lysine residues of histone tails helps transcription factors access the DNA in chromatin (Vettese-Dadey et al, 1996) by weakening internucleosomal interactions and destabilize higher-order chromatin structure (Garcia-Ramirez et al, 1995; Luger et al, 1997; Tse et al, 1998). Histone acetylation change high order chromatin structure and created a permissive local environment for the subsequent assembly of an active preinitiation complex (PIC) at the promoter (Martens et al, 2002; Struhl, 1998). For example, the recruitment of HAT CBP is prerequisite for the recruitment of Brg-1 – a component of the SWI/SNF chromatin remodeling complex (section 1.15) – in virus-induced IFN β promoter activation (Agalioti et al, 2000).

Collectively, these effects facilitate the formation of transcription complexes involving RNA pol II and hence the initiation of gene transcription (Nightingale et al, 1998). For example, the level of histone H3K9 acetylation is high at active gene promoters or the regions surrounding transcription start sites (TSSs) (Barski et al, 2007; Bernstein et al, 2005; Roh et al, 2007).

1.14 Histone methylation

Histone methylation occurs at several lysine and arginine residues and is linked to either gene induction/activation or gene repression, which depends on the residues affected and the number of methylated amino groups. For example, the levels of H3K4me1 and H3K4me2 was positively correlated with transcriptional levels whilst H3K27me3 levels were higher at silent promoters than at active promoters (Barski et al, 2007; Lee et al, 2006). Genome-wide analyses have revealed a typical pattern of histone methylation at promoters, enhancers and transcribed regions. From these studies, the monomethylation of H3K9, H3K27, H4K20, H3K79 and H2BK5 is correlated with gene activation, while on the contrary, the trimethylation of H3K9, H3K27, H4K20 and H3K79 is linked to gene repression (Barski et al, 2007). Selected histone modifications of relevance to the present study are considered further below.

1.14.1 H3K4me3

H3K4 methylation could mark a gene either for the recruitment of protein complexes related to transcription activation and/or for the removal of complexes involved in transcriptional repression, such as histone deacetylases. H3K4me3 is a highly conserved histone modification, and is mainly distributed to gene promoters and TSS regions, as well as regions associated with high CpG content and GC dense DNA (Calo & Wysocka, 2013; Koch & Andrau, 2011; Pekowska et al, 2011). H3K4me3 is always described as a positive histone marker associated with active or poised transcription (Barski et al, 2007; Bernstein et al, 2005; Roh et al, 2007). In mammalian cells, H3K4me3 can be catalyzed by methyltransferase MLL1, MLL2 and SETD1 (Katada & Sassone-Corsi, 2010; Zhang et al, 2015). The recruitment of these methyltransferases to target genes requires cell type-specific transcription factors and coactivators (Narayanan et al, 2007; Okuda et al, 2014).

H3K4me3 is commonly considered a positive mark in gene activation because it is essential for recruitment of other histone-modifying enzymes that are important during transcription initiation. For example, H3K4me3 is found to co-localize at active gene promoters and TSS regions with H3/H4 acetylation, and promotes acetylation of downstream H3/H4 through the recruitment of HATs (Bian et al, 2011; Hung et al, 2009). However, the role of H3K4me3 in transcription is complicated because H3K4me3 seems not always related to active transcription: H3K4me3 was also shown to suppress transcription on a subset of genes (Howe et al, 2017). Therefore, the ability of H3K4me3 to activate or repress transcription must depend on other factors, such as chromatin remodeling complex recruitment and the affinity of interaction, the other pre-existing histone modifications at the site, or the duration of its residency (Howe et al, 2017).

Currently, an important issue in understanding the correlation between gene activity and H3K4 trimethylation is whether the deposition of this modification is a cause or simply a consequence of the transcription state present at a particular gene. A time-resolved experiment showed that the peak of H3K4me3 deposition

lagged behind mRNA transcription in the majority of yeast genes that were involved in metabolic cycles. This observation suggested that the H3K4me3 deposition is unlikely to regulate transcription initiation on these genes. There are also studies that suggest that H3K4me3 might function in a post-initiation process, such as the commitment to splicing of nascent pre-mRNA (Mikkelsen et al, 2007), transcription termination, or maintaining transcription memory and consistency (Martin et al, 2006; Terzi et al, 2011). All these data seem to suggest that deposition of H3K4me3 is a consequence of transcription. However, many genes, including developmental regulators such as Hox genes, show reduced the recruitment of RNA polymerase II and reduced expression following the loss of H3K4 methylation which would indicate that H3K4me3 is a cause of transcription rather than a consequence (Wang et al, 2009). Collectively however, these findings all indicate that H3K4 methylation has a specific and non-redundant role in gene regulation.

1.14.2 H3K9me3

Histone H3 lysine 9 trimethylation (H3K9me3), also a conserved modification, can be catalyzed by multiple methyltransferases (HMTase) including SETD1 (or ESET), SUV39H1, SUV39H2, EHMT1(GLP) and EHMT2(G9A) (Kim & Kim, 2012). Normally, H3K9me3 and its HMTase play an essential role in gene silencing. In mammals, H3K9me3 is predominantly enriched in heterochromatin, especially in the non-genic regions including the pericentric DNA, long terminal repeats of transposons, interspersed repetitive satellite DNA and centromeric and plays an important role in heterochromatin compaction, chromatin stability and gene repression (Becker et al, 2016; Nizialek et al, 2016). However, trimethylated H3K9 is not restricted just to the heterochromatin, this modification also occurs at the promoters of some euchromatic genes that are related to development and lineage-specific functions (Hawkins et al, 2010; Tachibana et al, 2002).

Chromatin that is enriched in H3K9me3 provides a docking site for the recruitment and assembly of repressive complexes including heterochromatin protein-1(HP1) and HDACs. The removal of acetylation of H3K9, a positive

transcriptional activation marker, by HDAC results in further H3K9 methylation and enhanced gene repression (Lachner & Jenuwein, 2002; Shilatifard, 2006). Unlike the repressive function of H3K27me₃, the incorporation of which leaves the promoter accessible to general transcription factors, an H3K9me₃-marked promoter totally precludes the DNA from binding diverse transcription factors (Becker et al, 2016).

In apparent contradiction to its role in promoting heterochromatin formation and gene silencing, H3K9me₃ was also reportedly linked with transcription activation because an H3K9me₃ signal was detected at active chromatin domains in a genome-wide study (Barski et al, 2007). In addition, H3K9me₃ and HP1 γ were found enriched in the coding region of active genes (Vakoc et al, 2006) and their deposition relied on RNA polymerase II (pol II) elongation. These findings highlight the complexity of the histone modification code for the control of transcription.

1.15 Chromatin remodeling complex SWI/SNF

Mammalian DNA is tightly packed into chromatin and this packaging of DNA can have a repressive effect on gene expression by preventing the binding of specific transcription factors and the general transcription machinery to specific DNA binding sites. Therefore, opening chromatin structure to expose the DNA sequence for the binding of such factors is a critical step for the activation of gene transcription. In addition to changes in histone modification as just discussed, this can involve the recruitment of ATP-dependent chromatin remodeling complexes such as SWI/SNF.

SWI/SNF is one of the best studied nucleosome remodeling complexes. The SWI/SNF complex uses the energy of ATP hydrolysis to alter nucleosome structure and then to slide and transfer the nucleosomes to neighbouring DNA, or to destabilize and then evict histones from DNA (Peterson & Workman, 2000; Wu et al, 2009). In eukaryotic cells, several subunits of SWI/SNF complex were encoded by different gene families, which can then assemble to generate diverse

combination in different tissues (Wang et al, 1996a; Wang et al, 1996b). Various SWI/SNF complexes with different components have been reported in human cells and display cell type-specificity (Wang et al, 1996a; Wang et al, 1996b)

In humans, there are major two classes of the SWI/SNF complex: the Brg-1 (Brahma-related gene 1) based complex and the hBRM (brahma)-based complex (Wang et al, 1996a), defined according to the presence of ATPase subunit Brg-1 or BRM. These two complexes share most of the other core subunits including BAF155, BAF170, BAF47 and BAF57, and auxiliary subunits BAF53, BAF60, BAF45 and β -actin (Figure 1.5) (Hohmann & Vakoc, 2014; Wilson & Roberts, 2011)

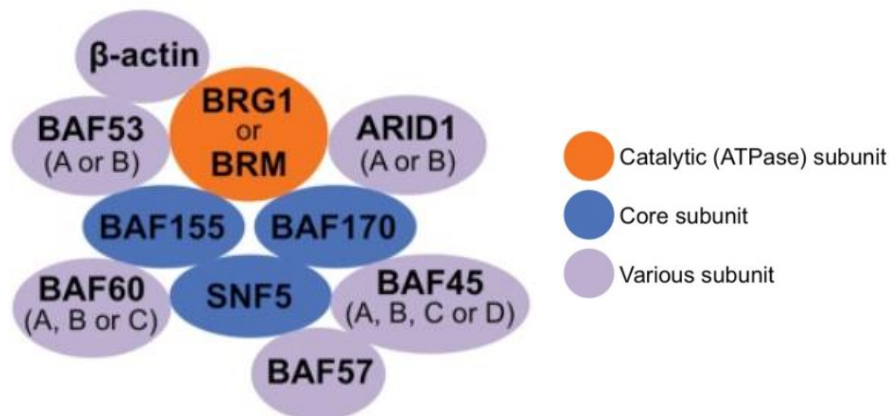


Figure 1.5 SWI/SNF complex

As the subunits of SWI/SNF are heterogeneous, the protein level of each subunit is stringently controlled via protein-protein interactions and proteasome-related degradation (Chen & Archer, 2005). The protein level of one subunit can affect the amount of other subunits. For example, the loss of BAF155 and BAF170 results in the degradation of BAF57, Brg-1 and BAF47; exogenous expression of BAF57 results in the downregulation of endogenous BAF57; and increased BRM protein level in HeLa cells by transient transfection results in a decline of endogenous Brg-1 (Chen & Archer, 2005).

Genome-wide studies of SWI/SNF interaction with chromatin have demonstrated that the complex prefers to bind transcription start sites (TSSs), enhancers and

many regions occupied by Pol II (Euskirchen et al, 2011). Although there was no evidence that binding of any particular form of the complex preferred at specific sites, increasing data showed that one or more subunits may play leading roles in SWI/SNF binding, which is depend on the stage of transcription, nucleosomal composition, and promoter architecture (Hogan & Varga-Weisz, 2007; Mohrmann & Verrijzer, 2005; Trotter & Archer, 2008). SWI/SNF complexes have several subunits that contain motifs that are able to bind DNA without sequence specificity, such as the AT-rich interactive domains of BAF250A (ARID1A), BAF250B (ARID1B), and BAF200 (ARID2); High Mobility Group (HMG) domains of BAF180 and BAF57 are also known to bind DNA without sequence specificity.

Three models of SWI/SNF recruitment to DNA specific sites have been proposed (Peterson & Workman, 2000). The first one is SWI/SNF is associated randomly on the chromatin templates and becomes targeted to a specific region of the chromatin in the presence of a gene-specific transcription factor (Owen-Hughes et al, 1996). The second model is the holoenzyme model in which SWI/SNF recruitment depends on RNA polymerase II (RNA pol II) holoenzyme, which targets SWI/SNF to the gene promoter through association with it (Peterson & Workman, 2000). The third 'Activator Model' is the most widely accepted. This model proposes that many gene-specific activators recruit SWI/SNF to its targets. The interaction of SWI/SNF complex with a transcription factor facilitates the recruitment of SWI/SNF to specific chromatin sites (Kadam & Emerson, 2003).

The presence of SWI/SNF was previously thought to indicate activation of transcription. However, more and more evidence indicates that SWI/SNF complexes are also involved in transcription repression and that this function can be either ATPase dependent or independent (Dunaief et al, 1994; Stankunas et al, 2008; Zhan et al, 2011). The ATPase-dependent repression activities may involve remodeling chromatin for the recruitment of transcription repressor or silencer, or to preclude the binding of activators. The ATPase-independent transcription repression is considered to occur through the formation of a

complex with DNA methylation enzymes or HDACs (Jani et al, 2008; Zhan et al, 2011).

1.16 Brg-1 and the IFN response

1.16.1 Brg-1

Brg-1 protein is one of the two ATPases in the SWI/SNF remodeling complex, and is encoded by the SMARCA4 gene in human. Its structure can be divided into three parts: a conserved C-terminal domain included a bromodomain and AT-hook motif, a catalytic ATPase region and a less characterized N-terminal region that encompasses a glutamine-leucine-glutamine motif (QLQ), as well as helicase/SANT-associated (HSA) and KIS (BRK) motifs (Trotter & Archer, 2008). These motifs have been identified to contribute to the recognition of modified histone proteins and/or to recruit the chromatin remodeling activity of Brg-1 to genomic targets.

Brg-1 is capable of providing the ATPase activity needed to remodel the chromatin during the transcription process, although most other SWI/SNF subunits are also required for the *in vivo* function of SWI/SNF complexes. *In vitro* studies showed that Brg-1 alone was sufficient to remodel the chromatin. However, the addition of core SWI/SNF subunits BAF170, BAF155 and BAF147 reconstituted chromatin remodeling activity to near optimal levels (Phelan et al, 1999).

As the central enzymatic subunit of SWI/SNF complex, Brg-1 plays important roles in transcription regulation. Brg-1 was implicated in various cellular activities by regulating the expression of a subset of genes associated with controlling related cell activities. For example, Brg-1 positively regulates CD44, MMP-2, MMP-9 and p53-dependent gene expression and cooperates with Rb to repress E2F1-mediated activation (Trouche et al, 1997), and negatively regulated the levels of CDK2, cyclin A and cyclin E (Reisman et al, 2002; Roberts & Orkin, 2004).

Many previously reports showed that Brg-1 recruitment to its target required gene specific transcription factors because Brg-1 lacks gene-specific DNA binding regions (Cosma et al, 2001; Cosma et al, 1999). This suggested that the Brg-1 recruitment is a secondary event in gene activation. However, in some genes such IFITM1, IFITM3 and CIITA (class II transactivator), Brg-1 was found bound on those gene promoters before IFN treatment (Cui et al, 2004; Liu et al, 2002; Ni et al, 2005), i.e. before the presumed arrival of gene-specific TFs. The mechanism for these observations is still not fully understood.

1.16.2 Brg-1 and IFN

Brg-1 is considered to be a transcription co-regulator essential for SWI/SNF chromatin remodeling complex to mediate transcription. In this role, Brg-1 has been reported to affect interferon responses. Brg-1 was shown to directly regulate expression of IFN β (Agaloti et al, 2000) and multiple ISGs (Cui et al, 2004; Huang et al, 2002; Pattenden et al, 2002). Brg-1 is required for the active expression of a subset of ISGs such as IFN α -induced transmembrane protein 1 (IFITM1), IFITM2, IFITM3, IFI27, guanylate-binding protein 1 (GBP1). The expression of IFN- γ -induced CIITA, MHCII, TRIM22, and IL-6 expression induced by LPS, was also impaired in cells that lacks Brg-1 (Ramirez-Carrozzi et al, 2006; Torti, 2012).

The binding of IFN α -induced ISGF3 complex to the promoters of several ISGs such as GBP1, IFI27 and IFITM1 is Brg-1-dependent (Ni et al, 2005). Mechanistic studies showed that Brg-1 absence does not impair STAT or IRF1 activity or expression but influences their binding to target gene promoters (Ni et al, 2005). In addition, Brg-1 binds specifically with both STAT1 and STAT2, and the binding of Brg-1 with STAT2 is independent of IFN α treatment. Brg-1 is also required for the expression of MHC class II and GBP1 in response to IFN- γ stimulation, because in a Brg-1/BRM-deficient cell line (SW13), IFN- γ failed to induce the expression of MHC class II and GBP1, a defect that was reversed by Brg-1 expression (Pattenden et al, 2002). The recruitment of STAT1 and IRF1 to the CIITA locus was also blocked upon IFN- γ stimulation in the absence of Brg-1 (Ni et al, 2008; Pattenden et al, 2002).

1.17 BRM and BAF

1.17.1 BRM

The human BRM (homologue of the *Drosophila melanogaster* brahma and *Saccharomyces cerevisiae* SNF-2 proteins) is another ATPase core component of the SWI/SNF complex. BRM is homologous to Brg-1, with around 74% identity in amino-acid sequences. Although Brg-1 and BRM belong to two separate forms of SWI/SNF complex, these complexes share most of their other subunits, therefore the functions observed for Brg-1 and BRM as they act within these complexes are partly similar and redundant. For example, both BRM and Brg-1 play an important role in ISG expression, and can activate IFITM3 promoter activity (Torti, 2012). They are both involved in retinoblastoma (Rb)-mediated signaling and BRM can compensate for Brg-1 function if Brg-1 is lost (Strobeck et al, 2002). The overall amount of available ATPase subunit is regulated as overexpression of BRM by transient transfection results in the decreased expression of endogenous Brg-1.

Despite their sequence similarity and functional interchangeability, BRM and Brg-1 are different in their regulated expression levels. Compared to Brg-1, which displays a relatively constant protein level in various cell types, the concentration of BRM protein is low at embryonic stem cells (ESCs) while is increased in differentiated cells (Kaeser et al, 2008; Muchardt et al, 1998). BRM and Brg-1 complexes regulate different gene expression profiles through selective association with certain classes of transcription regulator proteins. Zinc finger family of proteins interact exclusively with BRG1, while two ankyrin-repeats proteins CBF-1 and ICD22, that are essential in Notch signal transduction, are specifically associated with BRM (Kadam & Emerson, 2003). In addition, the requirements for BRM and Brg-1 in some ISGs expression is distinct. BRM is required for the basal expression and IFN α -induced IFIT1, IFIT3 and OAS1 expression, while Brg-1 is not important for the basal expression but important for the IFN α -stimulated expression of these genes (Lavigne et al, 2009). Thus BRM and Brg-1 can act synergistic or separately, or even antagonize each other, to regulate selective gene expression depending on the cell type and specific context.

1.17.2 BAF155

The importance of core SWI/SNF subunits such as BAF170, BAF155 and BAF147 for achieving optimal Brg-1 chromatin remodeling activity *in vitro* (Phelan et al, 1999) suggests important roles of at least some BAF subunits in promoter-specific targeting or to convey a stabilized nucleosomal conformation favorable for SWI/SNF activity (Phelan et al, 1999; Trotter & Archer, 2008). BAF155 is one of the core subunits of SWI/SNF remodeling complex, and is encoded by the SMARCC gene. BAF155 is highly homologous to BAF170, and is likely exist either as heterodimers (BAF155/BAF170) or as homodimers (BAF155/155) in the cell (Chen & Archer, 2005; Wang et al, 1996b).

BAF155 is essential to maintain the integrity of SWI/SNF complex (Chen & Archer, 2005; Sohn et al, 2007). Thus, BAF155 is believed to function as a scaffold protein to control the assembly and maintain the stability of SWI/SNF complex. The downregulation of BAF155 and its homologue BAF170 reduces the protein level of other major subunits of SWI/SNF such as Brg-1, BAF57, BAF47, and BAF60a, while overexpression of BAF155 increases their level. BAF155 directly associates with those subunits and protects them from protease degradation. The observation that the deletion of either Brg-1/BRM or BAF47 does not affect the interaction of remaining subunits with BAF155(Doan et al, 2004), while the absence of BAF155 impairs the stability of SWI/SNF complex, further supports the scaffold role of BAF155. (Trotter & Archer, 2004; Wang et al, 1996a). However, there is a study which showed that BAF155 was able to function independent from the full SWI/SNF complex, and seems BAF155 can bind at some genomic sites independent of Brg-1 (Wang et al, 2016).

1.18 Aims of this study

Recent work in our laboratory has demonstrated that PML isoform II (PML-II) regulates IFN functional responses and ISG gene transcription mainly by regulating transcription factors and/or coactivator recruitment at inducible gene promoters (Chen et al, 2015). As the distinct function of PML isoforms was thought to result from their different C-terminal structures, this suggested PML-

II C-terminal fragments should play an important role in PML-II transcription regulation functions. Therefore, the first aim of this study was to investigate PML-II C-terminal functional fragment in gene transcription.

PML protein binds with various TFs and co-activator CBP, and mediates the transcriptional complex recruitment at promoters (Chen et al, 2015). These findings suggested that PML-II may be a master regulator of gene expression having a general regulation function in chromatin remodeling and/or histone modification. Chromatin remodeling complex SWI/SNF was reported to regulate IFN responses (Agalioti et al, 2000) and ISG expression (Huang et al, 2002; Pattenden et al, 2002), so the second aim of this study was to elucidate PML-II regulation functions in chromatin remodeling and histone modification.

IFN α has been used to induce cell death/apoptosis in various malignant cancer cells (Ferrantini et al, 2007; Rizza et al, 2010). Several studies have shown that PML is required for IFN α to effectively inhibit cell growth or to induce apoptosis in various cancer cells (Crowder et al, 2005; Quignon et al, 1998; Wang et al, 1998). However, the exact mechanism remains to be completely understood. Therefore, the third aim of this study was to dissect PML-II functions in IFN α -mediated apoptosis.

Chapter 2 Materials and methods

2.1 Materials

This section lists the collections of biochemical reagents, antibodies, plasmids, siRNA sequences, qPCR primers, ChIP-qPCR primers that were utilized in this study.

Table 2.1.1 Chemical reagents and suppliers

Biochemical reagent	Supplier
Anti-FLAG M2 Affinity Gel	Sigma
Brilliant III Ultra-Fast SYBR Green qPCR Master Mix	Agilent Technologies
DMEM (41965)	Gibco
ECL prime (WB substrate)	GE healthcare
GenElute Mammalian Total RNA Miniprep Kit	Sigma-Aldrich
Lipofectamine 2000	Invitrogen
MG132	Sigma-Aldrich
PageRuler Plus Prestained Protein Ladder	Thermo scientific
poly(I:C)	Sigma
Protein A Sepharose CL-4B beads	GE Healthcare
Proteinase K	Sigma-Aldrich
PureYield Plasmid Midiprep system	Promega
QIAprep Spin Miniprep Kit	QIAGEN
Random Primers	Promega
IFN α	Pbl interferon source
	Ambion by Life
siRNA	Technologies
SYBR green PCR Master Mix	Agilent Technologies
TRI reagent	Sigma
Glycogen	Invitrogen
	Santa Cruz
Brg-1 si RNA(h):sc29827	Biotechnology
protease inhibitor cocktail	Sigma-Aldrich
phosphatase inhibitor cocktail	Sigma-Aldrich

Table 2.1.2 Antibodies

Antibody	Supplier
NF-κB p65(C-20): sc372	Cruz Biotechnology
CBP(C-20): sc-583	Santa Cruz Biotechnology
STAT1 p84/p91(E-23):sc-346	Santa Cruz Biotechnology
Mouse anti-PML-II	Millipore
Rabbit anti-Flag epitope	Polyclonal F7425 Sigma-Aldrich
Goat anti-mouse IgG-HRP	Sigma-Aldrich
Goat anti-rabbit IgG-HRP (sc-2054)	Santa Cruz Biotechnology
Rabbit anti-Brg-1 antibody(sc-4227)	Santa Cruz Biotechnology
mono clone mouse anti-Brg-1 antibody	Abcam
Rabbit anti-Brg-1 antibody(sc-10768)	Santa Cruz Biotechnology
Rabbit anti-BAF155 antibody	Santa Cruz Biotechnology
Rabbit anti-H3K4me3 antibody	Abcam
Rabbit anti-H3K9me3 antibody	Abcam
Rabbit normal IgG	Santa Cruz Biotechnology
Mouse normal IgG	Santa Cruz Biotechnology
Mouse anti-GAPDH antibody	Santa Cruz Biotechnology
AKT(s9272)	Cell signaling
p-AKT(Ser473)(s9271)	Cell signaling
Phospho-p70 S6 Kinase (Thr389) (108D2)9234	Cell signaling
ERK p44/p42	Cell signaling
p-p44/p42MAPK (Thr202/Tyr204)	Cell signaling
MEK1/2	Cell signaling
phospho-MEK1/2(Ser217/221)(41G9)	Cell signaling
Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb	Cell signaling
TRAIL (C92B9) Rabbit mAb-3219	Cell signaling
PUMA Antibody (4976)	Cell signaling
p53 (FL-393) sc-6243	Santa Cruz Biotechnology
Phospho-p53 (Ser15) 9284	Cell signaling
β-actin	Millipore

Table 2.1.3 Cell lines and bacteria

Cell line	Phenotype/Origin
HeLa	Supplied by Prof. M.A.McCrae, University of Warwick
HEK293	Human embryonic kidney cells expressing adenovirus type 5 E1A and E1B genes, supplied by Dr. K. N. Leppard, University of Warwick (Graham et al, 1977).
DH5 α	<i>Escherichia coli</i> K12, genotypes: <i>SupE44, ΔlacU169</i> (ϕ 80lac Z Δ M15), <i>hsdR, recA1, endA1, gyr96, thi-1, relA1</i>

Table 2.1.4 siRNAs

siRNA	Sense 5'→3'	Antisense 5'→3'	References
Control siRNA	GAGCCGGACGCCAAAGAAAUU	UUUCUUUGGCGUCCGGCUCUU	This thesis
PML-II siRNA	CAUCCUGCCCAGCUGCAAAUU	UUUGCAGCUGGGCAGGAUGUU	(Kumar et al, 2007)

Table 2.1.5 qPCR primers

Gene	Forward 5'→3'	Reverse 5'→3'	Reference
IFN β	ATTGCCTCAAGGACAGGATG	GGCCTTCAGGTAATGCAGAA	(Shi et al, 2010)
ISG15	CCCTCGAAGGTCAGCCAGA	GGACAAATGCGACGAACCTCT	(Han et al, 2011)
ISG54	TGCAACCTACTGGCCTATCTA	CAGGTGACCAGACTTCTGATT	(Shi et al, 2010)
ISG56	GCCATTTTCTTTGCTTCCCCTA	TGCCCTTTTGTAGCCTCCTTG	(Yang et al, 2009)
PML-II-1+2	AGGCAGAGGAACGCGTTGT	GGCTCCATGCACGAGTTTTC	(Chen et al, 2015)
PML-II-3+4	GAAAACTCGTGATGGAGCC	GAGGCTCCTTGTGCTCTCAG	This study
GBP1	CAAGCTAAAGCAAACCACCTT	TGGTTTCTTCCACCTTCCTC	This study
OAS1	TGCGCTCAGCTTCGTA CTGA	GGTGGAGTTCTCGCCCTCTT	This study
IFITM3	TTTGTTCCGCCCTCATCTG	CTTTTCCTGGCATTGTGCAAATC	This study
IFI27	CAAGTTCATCCTGGGCTCCAT	GGGCAGGGAGCTAGTAGAACCT	This study
Brg-1	GTTTGAGGACACCAT TGACCATA	AATGCCAAGCAAGATGTCGAT	This study
BRM	TGGAAATTCTGCAAGAGCGG	CCACGGTTGCTTTGGTTCTTAA	This study
BAF155	GCGAGTTCTACATGATCGGCT	CACAGTGGCTAGTCGCCTC	This study
BAF47	GGTCGGAGTCAACGGATTT	CCAGCATCGCCCCACTTG	This study
c-Fos	CCGGGGATAGCCTCTCTTACT	CCAGGTCCGTGCAGAAGTC	This study
c-Myc	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT	This study
PUMA	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT	This study
TRAIL	TGCGTGCTGATCGTGATCTTC	GCTCGTTGGTAAAGTACACGTA	This study
p53	GCCCAACAACACCAGCTCCT	CCTGGGCATCCTTGAGTTCC	This study
BAK	ATGGTCACCTTACCTCTGCAA	TCATAGCGTCGGTTGATGTCG	This study
Bcl2	GGTGGGGTCATGTGTGTGG	CGGTT CAGGTA CT CAGTCATCC	This study

Table 2.1.5 qPCR primers (continue)

Gene	Forward 5'→3'	Reverse 5'→3'	Reference
Bcl-xL	GAGCTGGTGGTTGACTTTCTC	TCCATCTCCGATTCAGTCCCT	This study
GAPDH	GGTCGGAGTCAACGGATTT	CCAGCATCGCCCCACTTG	(Vestergaard et al, 2011)
β-actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT	(Shi et al, 2010)

Table 2.1.6 ChIP primers

Genes	Forward 5'→3'	Reverse 5'→3'	reference
IFNβ	TGCTCTGGCACAACAGGTAG	CAGGAGAGCAATTTGGAGGA	(Chen et al, 2015)
ISG15	CGCCACTTTTGCTTTTCCCT	ATAAGCCTGAGGCACACACG	(Chen et al, 2015)
ISG56	TTGGGTTTCTGCAGCACTAGA	ACCTAGGGAAACCGAAAGGG	(Chen et al, 2015)
ISG54	GCCGAACAGCTGAGAATTGC	CTGGCCCTCTTTGGGAACAT	This study
IFITM3	TCAGGAATTTGTTCCGCCCTC	GATTCATGGTGTCCAGCGAAGAC	This study
OAS1	CAGCAGTCCAAGCTCAGTCA	GCTACCTCGGAAGCACCTTT	This study
GBP1	TTAAACCCTCCCACTGGCAC	GAGCACTGGCTTCTAGCACT	This study
ISG56(promoter downward)	CTCTGCCTATCGCCTGGATG	CCTGCCTTAGGGGAAGCAAA	This study
ISG15(promoter downward)	ATCCTGCCTGTCCTCCATCT	AGGACTCACCCCTTACTGCT	This study
ISG15(promoter downward)	AGGCAGGCTCTCCATAGGAA	GTCTCTACCCCTCCCTAGCC	This study
ISG54(promoter downward)	CTGCCACCTGCTACCATTGA	GCATTTGGATTGCGTCCAGG	This study
PUMA	GCGAGACTGTGGCCTTGTGT	CGTTCCAGGGTCCACAAAGT	This study

2.2 Methods

2.2.1 Cell culture

HeLa cell, a cervical cancer cell line, and HEK293 cell, human embryo kidney cells immortalized by a fragment of adenovirus DNA (Graham et al, 1977), were grown in 10cm γ -irradiated tissue culture dishes and were maintain in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator. When the cells were confluent, they were passaged by washing the monolayers with 3 ml versene (0.02% EDTA in PBS), followed by trypsinizing with 2.5 ml versene containing 0.02% (v/v) 0.5ml trypsin. The detached cells were collected into 25 ml tube with 0.5 ml FBS and pelleted by centrifugation at 2000 rpm for 3 min. The cell pellet was then resuspended with DMEM / 10% FBS and seeded into new dishes at the required density for experiments or further passage.

2.2.2 Cloning/plasmid preparation

2.2.2.1 Transformation of competent bacterial cells

Plasmid DNA (typically 50 ng) was added into 50-100 μ L of thawed competent DH5 α and left on ice for 20-30 min. Cells were then placed at 42 °C water bath for 30s, followed by immediate incubation on ice for another 2-3 min. 500-800 μ L of pre-warmed liquid LB medium was added and the LB-competent cell mixture was incubated at 37 °C in a shaking incubator for 1 h. After that, the cells were plated onto LB plates containing 100 μ g/ml ampicillin to select for retention of plasmid and incubated at 37 °C overnight.

2.2.2.2 Extraction of plasmid DNA

Plasmid DNA was extracted using either the Qiagen MiniprepKit™, or the Promega Pureyield™ Plasmid Midiprep System, using the protocols provided by the manufacturers following their instructions. Before DNA was eluted from columns with nuclease-free water, the optional Endotoxin Removal Wash step was included to enhance removal of any endotoxin, protein, RNA or other contaminants.

2.2.2.3 Quantification of nucleic acid

The concentration of DNA and RNA samples was determined using a Nanodrop-ND1000 spectrophotometer (Thermo Scientific) by measuring OD₂₆₀. The purity of DNA and RNA was indicated by the absorbance ratio of OD₂₆₀ to OD₂₈₀, where a ratio of 1.8 indicates pure DNA and a ratio of 2 indicating pure RNA.

2.2.3 Transfection and stimulation

2.2.3.1 Plasmid DNA transfection

A concentration of 2×10^5 per ml HEK293 cells or 1.0×10^5 Hela cells were seeded in plates, respectively, and transfected after 24 h of culture. This concentration of cells were used in all DNA or RNA transfection experiments in this study. Lipid reagent LT1 (Mirus) was used for liposome-mediated transfection of plasmid DNA, with a ratio of 2 μ L LT1 per μ g plasmid DNA added. The transfection reagent and plasmid DNA was prepared in pre-warmed Opti MEM medium. After mixing, the transfection reagent/plasmid DNA complexes were incubation for 20 min at room temperature, then pipetted onto the cells in normal growth medium and gently mixed. Cells were cultured for various times depending on the different requirements of individual experiments.

2.2.3.2 siRNA (short interfering RNA) transfection

An optimized input of 125 pmol/ml siRNA was used for transfection in this study. Lipofectamine 2000 (Invitrogen) was the transfection reagent used and the ratio of siRNA to Lipofectamine 2000 was 100 pmol siRNA per microliter of Lipofectamine 2000. The transfection reagent and siRNA was prepared in pre-warmed Opti MEM medium, mixed and incubated at room temperature for 20 min, then gently pipetted onto the cells and gently mixed.

2.2.3.3 poly(I:C) and IFN α treatment of the cells

1.0 μ g/ml poly(I:C) or 1000U/ml IFN α was used to stimulate the cells. Lipofectamine 2000 (Invitrogen) was the transfection reagent used for poly(I:C) stimulation, with 1 μ g poly(I:C) added to 2 μ L Lipofectamine 2000. Lipofectamine 2000 and poly(I:C) were prepared in pre-warmed Opti MEM medium separately. After mixing, the transfection reagent complex was incubated for 20 min at room temperature, then pipetted onto the cells in normal

growth medium and gently mixed.

IFN α was diluted with pre-warmed Opti MEM medium and added into the cells directly. The length of poly(I:C) or IFN α stimulation varied depending on the different requirements of individual experiments.

2.2.4 RNA extraction and reverse transcription

Cells were harvested and lysed from 24-well-plate cultures, and RNA was extracted by using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions. RNA pellets were dissolved in 40 μ l sterile H₂O.

Before reverse transcription, the RNA was treated with DNAase to remove any contaminating DNA. Typically, 1 μ g of RNA was used per reaction and incubated with 1unit DNAase and 1 μ l 10 \times DNAase Reation Buffer (Promega) along with H₂O to a volume of 10 μ l at 37°C for 30 min, then 1 μ l DNAase stop buffer was added and incubated at 65°C for 10 min.

After DNAase treatment, the RNA was subjected to reverse transcription. The reactions were performed in a 20 μ L volume using GoScript™ reverse transcriptase according to the manufacturer's instructions. 5 μ L RNA was incubated with 1 μ L random primer at 70°C for 5 min then chilled on ice for 5 min. Then a mixture containing 4 μ L GoScript™ 5 \times reaction buffer (Promega), 2 μ L MgCl₂(25mM) 1 μ L PCR nucleotide mix (10mM), 20 units recombinant Rnasin ribonuclease inhibitor (Promega), 1 μ L GoScript™ reverse transcriptase (Promega)and 6 μ L H₂O was added to each sample, mixed and placed on the PCR machine. The reaction conditions included annealing 25°C 5 min, extending 42°C 1 h and then 70°C 15 min to inactivate the reverse transcriptase.

2.2.5 SYBR-Green quantitative PCR

For quantitative PCR (qPCR) reactions a SYBR-Green qPCR Master Mix was used according to the manufacturer's instructions (ABI or Agilent). Briefly, the reaction mixture contained 10 μ L SYBR green Master Mix, 0.2 μ L forward and reverse primers (10 μ M each primer) and 1 μ L (50-100 ng) DNA or cDNA with

H₂O to a total 20 µL volume was added into the wells of qPCR plates and qPCR amplification was carried out using a Stratagene Mx3005P light cycler (Agilent Technologies). qPCR primers used in this study are listed in Table 2.1.5.

Reaction conditions were: denaturation at 95°C for 10 min, then 40-50 cycles of denaturation at 95°C 30 sec, annealing/extension at 60°C for 60 sec. The results were analyzed using Agilent Technologies system software. Quantification was based on Ct difference performed according to the “delta–delta Ct method” (Livak & Schmittgen, 2001), using the following equation: expression ratio = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t \text{ target} - C_t \text{ reference})$. A standard curve was established by plotting the C(t) values obtained from the qPCR against the log of plate counts on control siRNA and showed good correlation. qPCR efficiency calculated from standard curves ($E = [10^{-1/\text{slope}}] - 1$). Target gene expression was normalized against expression of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin. All samples, including the no GoScript™ reverse transcriptase negative controls and non-template controls, were analysed in triplicate.

2.2.6 Western-blotting

Protein samples in SDS sample buffer (4% SDS, 20% glycerol and 50mM Tris.HCl (pH6.8)) were boiled for 5-10 min before loading onto SDS polyacrylamide gels and electrophoresis at 110 V for 1.5 h. Proteins were then transferred to a nitrocellulose membrane and the membrane then blocked with 2% (w/v) milk diluted with PBS containing 0.05% Tween 20 (PBS-T) at room temperature for 1 h, or at 4 °C overnight. The membranes were then incubated with the appropriate concentration of specific primary antibody (Table 2.1.3) diluted with blocking solution for 1 h at room temperature on an orbital shaker. The dilution of the primary antibody was dependent on the protein under investigation. Membranes were then washed with PBS-T 4 times, with the PBS-T being replaced every 10 min. After washing, the appropriate HRP-conjugated secondary antibody was added and incubated and washed as before. Finally, the

blot was detected using ECLTM Advance reagent (GE healthcare) according to the manufacturer's instruction by exposure to Fuji Super RX X-Ray film.

2.2.7 Flow cytometry

Cell death/apoptosis was determined by flow cytometry. Briefly, HeLa cells were cultured in 24-well plates. Following transfection and/or stimulation treatment, culture medium was removed, and cells were washed once with ice-cold PBS, released from the well with trypsin. Cells were collected and centrifuged at 1,100 rpm for 3 min, then washed twice with cold PBS. Cells were resuspended in PBS then stained by incubation with 100 μ l PBS containing 0.5–5 μ l (0.1–1 μ g) of Annexin-V FITC and/or 1 μ l propidium iodide (PI) (50 μ g/ml) and put on ice for 20–30 min in the dark. After twice washing with cold PBS, cells were transfected to FACS tubes for flow cytometry. Assays were performed in duplicate and data were acquired using a FACSCanto II flow cytometer (BD Biosciences).

2.2.8 Co-immunoprecipitation

Two alternative protocols were followed, either a standard antibody protocol or one specifically for Flag epitope tagged proteins.

Standard antibody protocol: Cells cultured in 10 cm dishes (80–100% confluent, about 6×10^6 – 1×10^7 cells) were washed once with 10 ml cold PBS. Then cells were scraped, lysed with 0.5–1.0 ml cold NP-40 cell lysis buffer (50 mM Tris.HCl pH8.0, 150 mM NaCl, 1% (v/v) NP-40) on ice for 10 min and sonicated 40 min using the Biorupter (Diagenode). After centrifugation in a microfuge at 13,000 rpm for 10 min, the sonicated cell lysate was incubated with specific antibody, by rotation at 4 °C overnight. Protein A Sepharose beads were then added and rotated for 2 h to collect antibody:antigen complexes. After washing the protein/antibody/beads complex with 0.5–1.0 ml IP-washing buffer (10 mM Tris.HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM EGTA and 0.2 mM Na_3VO_4) five times, the beads were resuspended with 2 \times SDS sample buffer and boiled for 10 min to release bound proteins. After centrifugation, the supernatant was recovered, 10 μ L DTT (dithiothreitol) added and stored for western blotting.

Flag beads fusion protein protocol: Cells cultured in 10 cm dishes (80-100% confluent, about 6×10^6 - 1×10^7 cells), were washed three times with 10 ml cold PBS and lysed on ice with 0.5 ml cold 25 mM HEPES (pH 7.0), 0.5 M NaCl, 0.1% NP-40, 1mM sodium butyrate, 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phosphatase inhibitor cocktail (Sigma-Aldrich) (Hoppe et al, 2006). Cell lysates were collected and sonicated on ice by the Biorupter for 20 min and then diluted with equal volumes of low salt lysis buffer (as above except 50 mM NaCl). Flag affinity gel was thoroughly resuspended and washed with TBS buffer and 0.1 M Glycine (pH3.5) following product instruction. The protein complexes were then precipitated with 50 μ l Flag affinity gel per sample at 4 °C for 3 h or overnight. After extensive washing with TBS buffer, 40 μ L 2 \times SDS sample buffer was added into the beads and boiled for 10 min before centrifugation at 13,000 rpm for 10 min in a microfuge. The supernatant of the mixture was recovered, 10 μ L DTT added and stored for western blotting.

2.2.9 Chromatin immunoprecipitation (ChIP) assay

Cells cultured in 10 cm dishes (80-100% confluent, about 6×10^6 - 1×10^7 cells) were cross-linked with formaldehyde to final concentration of 1% for 10 min at room temperature, then the reaction was stopped by the addition of glycine to a final concentration of 125 mM. After 5 min, fixed cells were washed twice with cold PBS, scraped from the dish and resuspended in Buffer C (20 mM HEPES pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) on ice for 20 min. Nuclei were pelleted by centrifugation at 13,000 rpm, 10 min in a microfuge, and lysed with 120 μ l Breaking Buffer (50 mM Tris-HCl pH8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS, 2% Triton X-100) on ice for 10 min, then sonicated with biorupter for 40 min to shear DNA to lengths between 200 and 1000 bp. Nuclear lysates were then diluted with 1 ml Triton buffer (50 mM Tris-HCl pH8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100) and stored in aliquots at -80 °C. Lysates were pre-cleared with 20 μ l Protein A Sepharose beads at 4 °C for 2 h to reduce non-specific binding, then precipitated by 1-2 μ g specific antibody at 4°C overnight. Antibody complexes were collected on 50 μ l Protein A Sepharose at 4°C for 3 h. The antibody/chromatin/bead complexes

were then washed with Triton buffer four times and then once with TE buffer (1M Tris-HCl pH 8.0, EDTA 0.5M), before the cross-linking was reversed by incubation with 400 μ l of SDS-NaCl buffer (62.5 mM Tris.HCL pH 6.8, 200 mM NaCl, 2% SDS, 10mM DTT) at 65°C overnight.

ChIP-DNA was then recovered and purified by the following protocol. Samples were digested with 0.02 mg Proteinase K, 5 mM EDTA and 20 mM Tris.HCL pH 6.5 for 1 hour at 45°C. 400 μ L phenol-chloroform (1:1) was then added to each sample, shaken and centrifuged at 13,000 rpm for 10 min at 4°C, collecting the upper aqueous phase. Phenol-chloroform (1:1) extraction was repeated, followed by chloroform extraction as before. DNA was precipitated by adding two volumes of 100% ethanol containing 0.3M Na acetate, 0.3 μ L glycogen at -20°C overnight. DNA was pelleted by centrifugation 13,000 rpm for 10 min, then the DNA pellets were washed with 70% ethanol, dried for 20-30 min in a vacuum dessicator, and finally dissolved in 40 μ l of H₂O.

ChIP-qPCR assay was carried out using SYBR-Green qPCR Master Mix and specific primers as described in Section 2.2.5. CHIP qPCR primers used in this study are listed in Table 2.1.6. The relative ChIP signal of each gene target in a recovered ChIP DNA sample was expressed as % input specifically precipitated, with input DNA being quantified in parallel from DNA purified from a sample of chromatin reserved at the nuclear lysate stage.

Chapter 3 PML-II C-terminal is essential for its transcription regulation function

3.1 Introduction

Multiple isoforms of the PML protein are involved in many cellular activities including gene transcription regulation (Chen et al, 2015; Zhong et al, 2000b). Different isoforms have distinct functions due to various C-terminal functional structures which are important for their interaction with partner proteins (Bernardi & Pandolfi, 2007; Geng et al, 2012; Nisole et al, 2013). Previous work in our laboratory demonstrated that PML isoform II (PML-II) is specifically required for efficient induction of IFN β transcription and of numerous ISGs (Chen et al, 2015). PML-II regulates the expression of these genes through affecting transcription factor recruitment and transcriptional complexes binding at the promoter of these genes (Chen et al, 2015). Because it is the distinct C-terminal domain that distinguishes PML-II from other isoforms, it was presumed that the distinct function of PML-II resided in that C-terminal structure. This idea implies that sequences in the PML-II C-terminal domain may be essential for PML-II to bind to transcription factors and to be recruited to target gene promoters, thereby exerting a distinct function in transcriptional complex assembly at the promoter. This chapter tests this hypothesis. Some of the data in this chapter have been published as part of a collaborative paper (Chen et al, 2015).

3.2 Results

3.2.1 The unique C-terminal domain of PML-II is essential for binding with TFs and functions.

Previous work in our laboratory has demonstrated that a 40 aa residue segment from the C-terminal domain of PML-II confers an interaction between this protein and Ad5 E4 Orf3 (Leppard et al, 2009). It was therefore important to test whether these potential protein interaction sites from the unique C-terminal of PML-II could also interact with cellular components such as transcription factor (TF).

To address this, full length PML-II and three available deletion mutants PML-II- $\Delta 1$, PML-II- $\Delta 2$ and PML-II- $\Delta 3$ were tested for TF binding by co-immunoprecipitation assay. These mutants were constructed by deleting specific conserved regions within the C-terminal domain of PML-II based on sequences alignment, see figure 3.2.1A (Leppard et al, 2009). As expected, full-length PML-II could bind with transcription factor NF- κ B and STAT1 (Figure 3.2.1 B). Deletion of $\Delta 1$ and $\Delta 2$ affected PML-II binding to NF- κ B transcription factors and slightly affected STAT1, while removal of fragment $\Delta 3$ had no effect on this binding ability (Figure 3.1 B). This result suggests that parts of the PML-II C terminal are essential for TF binding, specifically the regions covered by $\Delta 1$ and $\Delta 2$.

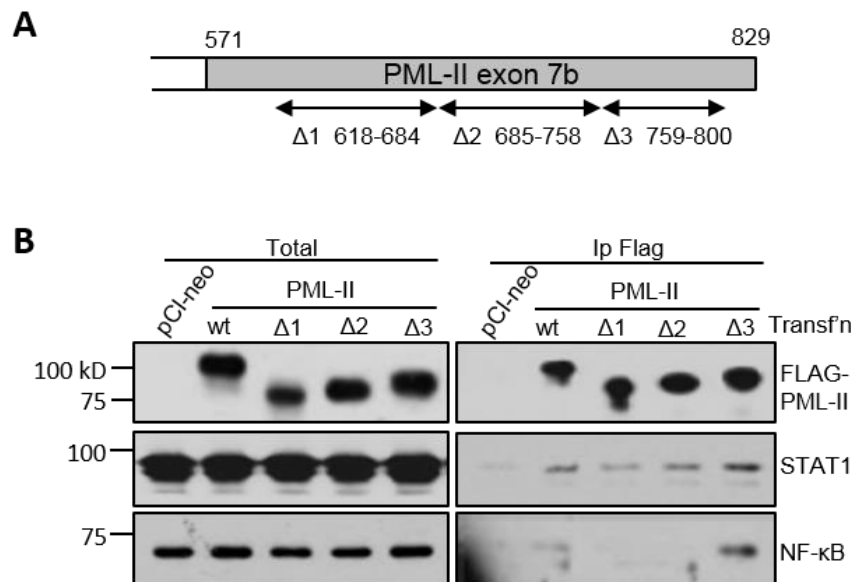


Figure 3.2.1 PML-II C-terminal is essential for its binding ability with STAT1 and NF- κ B (A) Representation of the C-terminal region of PML-II showing the positions of deletion mutations. (B) HEK293 cells were transfected with 250 ng/ml Flag-PML-II, Flag-PML-II- $\Delta 1$, Flag-PML-II- $\Delta 2$, Flag-PML-II- $\Delta 3$ plasmids or pCI-neo empty vector as indicated for 24 h, and then stimulated with poly(I:C) for 16 h, and lysates were prepared and immunoprecipitated with anti-Flag beads. Precipitates and total lysates were analyzed for FLAG-PML, NF- κ B (p65) and STAT1 by Western blotting.

3.2.2 PML-II C-terminal binding of TFs independent of N-terminal sequences

The N-terminal RBCC domain/structure of PML protein mediates a strong homo/ hetero-dimerization with other PML isoforms (Jensen et al, 2001). It is likely also to contribute to biochemical functions of the proteins. To determine whether the TF binding activity of PML-II required these sequences, and to exclude the possibility that PML-II binding with other isoforms, which might have an opposite or synergistic function, affected the PML-II contribution to the IFN β response, binding experiments were repeated using Δ RBCC forms of PML.

PML-V has been demonstrated in our laboratory to have no effect on IFN induction and downstream signaling, therefore full length PML-II and another isoform PML-V as well as their respective Δ RBCC mutant forms, PML-II- Δ RBCC and PML-V- Δ RBCC plasmids were used to test their interaction with STAT1 under IFN α stimulation. The result showed that both full length PML-V and PML-II were capable of associating with STAT1. However, the binding ability of PML-V with STAT1 was abolished if its N-terminal was deleted, while the deletion of N-terminal RBCC domain has less effect on PML-II binding with STAT1. These results suggested that PML-II specifically associates with STAT1, and that its C-terminal is necessary and sufficient for that association. In contrast, the observed interaction of full length PML-V and STAT1 is largely dependent on its N-terminal structure. This dependence suggests that the PML-V STAT1 interaction is indirect, the N-terminal RBCC domain allowing hetero-dimerization with other PML isoform such as PML-II that interacts directly with STAT1.

To further test this interpretation, the TF binding of the PML-II- Δ 1, PML-II- Δ 2 and PML-II- Δ 3 mutants was tested in a Δ RBCC context. As expected, the deletions Δ 1 and Δ 2 in the Δ RBCC context disrupted the binding of NF- κ B and STAT1, as was also seen with the full-length constructs, while deletion Δ 3 had no effect on the interaction (Figure 3.2.2B).

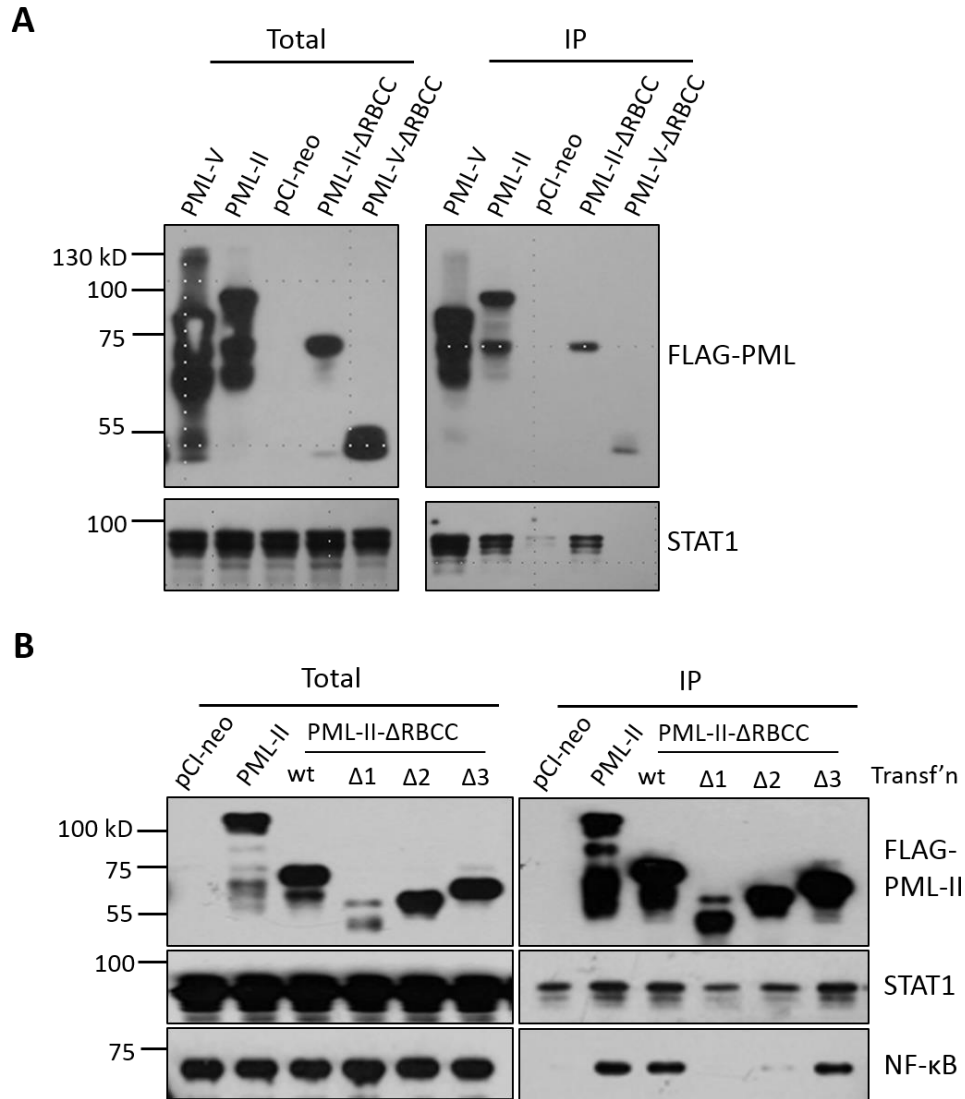


Figure 3.2.2 PML-II C-terminal binding TF independent of N-terminal. (A) HEK293 cells were transfected with 250 ng/ml Flag-PML-II, Flag-PML-V, Flag-PML-II-ΔRBCC, Flag-PML-V-ΔRBCC and pCI-neo empty vector, respectively, for 48 h and then cells were stimulated with IFN α for 10 h. The lysates were prepared and immunoprecipitated with anti-Flag beads. Precipitates and total lysates were analyzed for Flag-PML and STAT1 by Western blotting. (B) HEK293 cells were transfected with 250 ng/ml Flag-PML-II-ΔRBCC mutant or further deletion mutant plasmids Flag-PML-II-ΔRBCC-Δ1, Flag-PML-II-ΔRBCC-Δ2, Flag-PMLII-ΔRBCC-Δ3 or pCI-neo empty vector for 48 h and then cells were stimulated with poly(I:C) for 16 h, and lysates were prepared and immunoprecipitated with anti-Flag beads. Precipitates and total lysates were analyzed for Flag-PML, NF- κ B (p65) and STAT1 by Western blotting.

3.2.3 Residues of 645-665 are essential for PML-II binding with STAT1 and NF- κ B

In order to map more precisely the sequences of PML-II required to bind transcription factors STAT1 and NF- κ B, further plasmid mutants were tested which were constructed in the unique C-terminal domain of Flag-PML-II- Δ RBCC. It can be seen that the Δ m1 sequences (residue 645-665), especially regions removed in Δ D are essential for the PML-II interaction with NF- κ B, with almost no NF- κ B protein being precipitated by the Flag antibody in cells transfected with this plasmid. The Δ B and Δ C region within Δ m1 is also important for the binding of PML-II with NF- κ B. The deletion of both regions impaired the binding of PML-II with NF- κ B although with less effect compared to Δ D. In contrast to NF- κ B, the binding of PML-II with STAT1 was mainly focused at the regions deleted in Δ B, Δ C and Δ D. This suggested that different transcription factors have different binding sites in the C-terminal of PML-II. Taken together, these results suggested that PML-II can specifically associate with STAT1 and NF- κ B, and its C-terminal is necessary for these interactions.

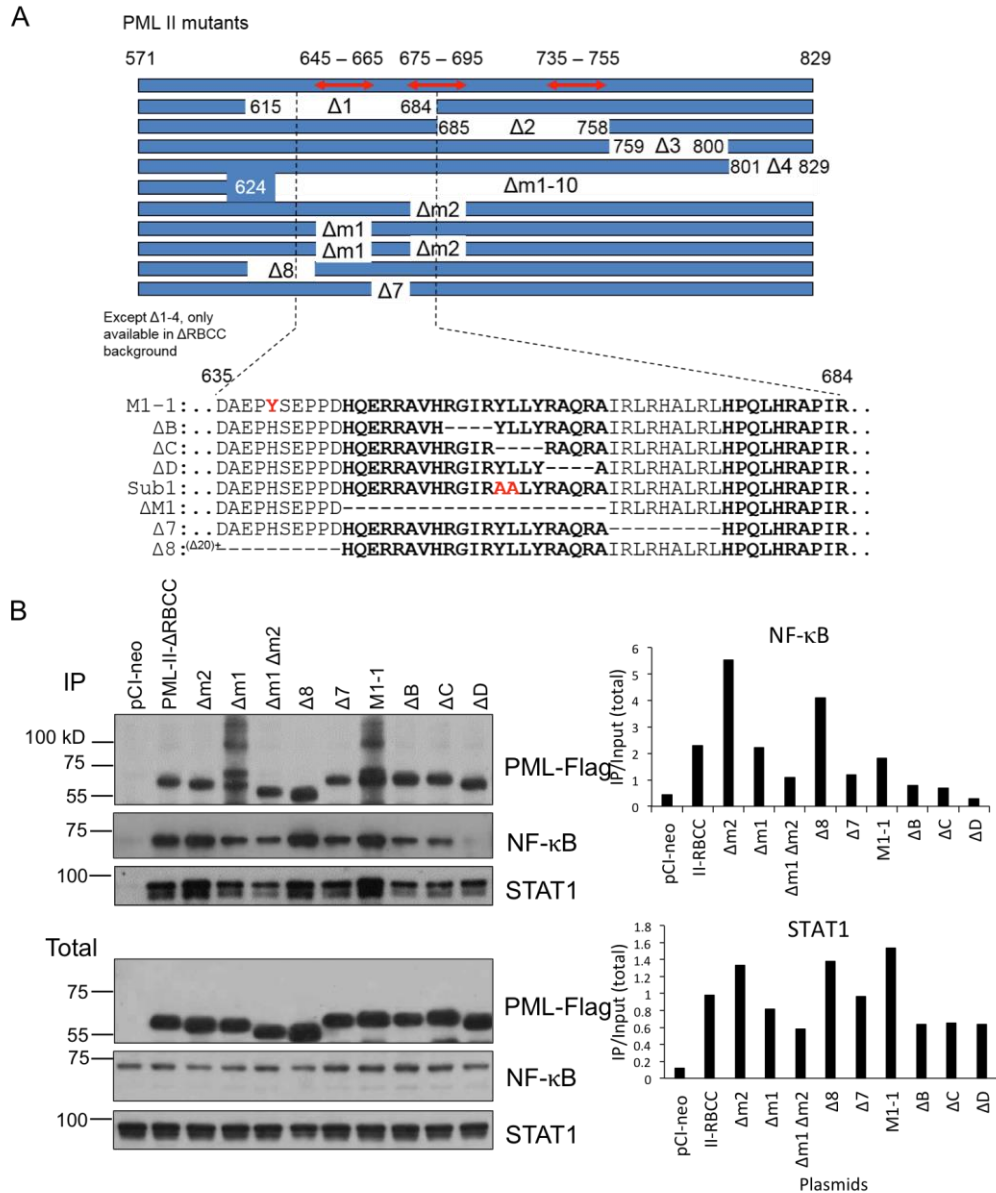


Figure 3.2.3 Residue of 645-665 is essential for PML-II binding with STAT1 and NF-κB. (A) The cartoon represents the unique C-terminal domain that defines PML-II (residues 571 – 829 of full-length PML-II), with the sequences deleted or altered in individual mutants indicated (Leppard et al, 2009). (B) HeLa cells were transfected with 250 ng/ml Flag-PML-II, Flag-PML-II-ΔRBCC, Δm1, Δm2, Δm1Δm2, Δ8, Δ7, M1-1, ΔB, ΔC, ΔD plasmids or pCI-neo empty vector for 24 h, then stimulated with poly(I:C) for 10 h, lysates prepared, immunoprecipated with anti-FLAG beads and precipitates & total lysates analysed for FLAG-PML, STAT1, and NF-κB by western blotting. (C) The precipitated NF-κB and (D) STAT1 bands in (B) were quantified and normalized to total expressed proteins (input).

3.2.4 Deleting the N-terminal RBCC domain of PML-II does not affect PML-II regulation function in a type I IFN response.

Having shown that TF binding was an autonomous function of the C-terminal of PML-II, it was important to test whether this binding with TFs correlated with function in an IFN response. Full length PML-II and its different mutants, PML-II- Δ RBCC, PML-II- Δ RBCC- Δ 1, PML-II- Δ RBCC- Δ 2, PML-II- Δ RBCC- Δ 3 and empty vector pCI-neo, were transfected into HEK293 cells and the effect of their overexpression on IFN β induction and on the downstream response were tested by looking at IFN β , ISG54 and IP-10 mRNA levels.

It can be seen that the overexpression of PML-II- Δ RBCC plasmid promoted ISG56 expression (Figure 3.2.4A). Although IFN α stimulation increase the expression level of ISG56, the level was further increased by overexpressing PML-II Δ RBCC. Similar results were also observed in the other genes including IFN β and IP-10 (Figure 3.2.4B and C). This suggested that the removal of the RBCC domain of PML-II alone did not impair function but actually further enhanced PML function in the expression of IFN β and ISGs in response to different stimulations (Figure 3.2.4 B and C). In contrast, overexpression of PML-II- Δ RBCC- Δ 1 and PML-II- Δ RBCC- Δ 2 reduced the expression of ISG56 compared to that of overexpressing PML-II- Δ RBCC, this suggests the deletion of C-terminal fragments in PML-II particular Δ 1 and Δ 2 may impair the function of PML-II in gene expression (Figure 3.2.4A). This is also consistent with the co-IP data that Δ 1 or Δ 2 is important for transcription factor binding with PML-II, suggesting an important function of Δ 1 and Δ 2 regions in PML-II-mediated transcription (Figure 3.2.4A). It is noticeable that the deletion of Δ 3 increased ISG56 expression, however the significance of this remains unclear (Figure 3.2.4A). Collectively, these results indicated that PML-II C-terminal was essential for its function, while loss of the N-terminal RBCC domain had no effect on PML-II regulation of type I IFN response.

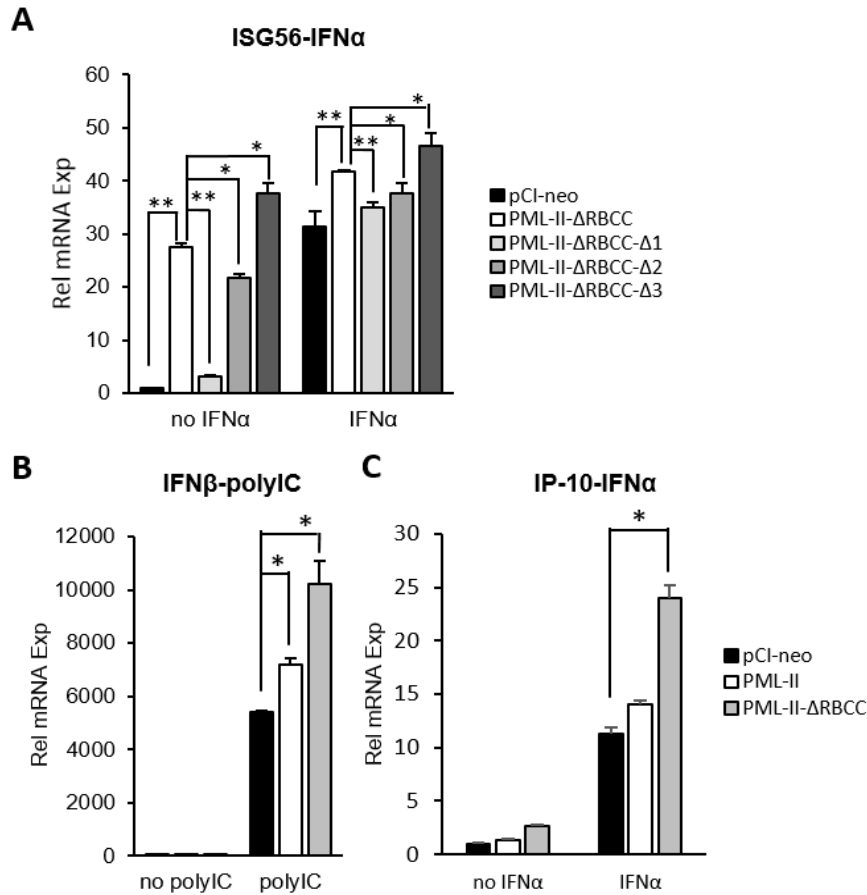


Figure 3.2.4 Deleting the N-terminal RBCC domain of PML-II does not affect PML-II regulation function in type I IFN response. HEK293 cells were transfected with 250 ng/ml Flag-PML-II- Δ RBCC, Flag-PML-II- Δ 1, Flag-PML-II- Δ 2, Flag-PML-II- Δ 3 plasmids or pCI-neo empty vector for 24 h. Following 1 μ g/ml 1000 U/ml IFN α or poly(I:C) stimulation for 16 h. Total RNA was analyzed for specific gene (A) ISG56, (B) IFN β (C) IP-10 mRNA levels by RT-qPCR. mRNA level was quantified by $\Delta\Delta$ Ct method and is displayed relative to those observed in empty vector pCI-neo transfected cells without stimulation. Data shown are the mean \pm SD of technique triplicate. Two tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

3.2.5 PML-II binds/is recruited to the promoter of inducible genes

PML-II is associated with multiple transcriptional complexes under the condition of stimulation, suggesting that PML-II might also be recruited to the promoter of the inducible genes and that such recruitment might be necessary for regulating

the transcription of inducible genes in response to various stress responses. My results had showed that PMLII- Δ RBCC had a stronger ability to bind transcription factors compared to full length PML-II. Moreover, using the PMLII- Δ RBCC form avoided effects of potential interactions between exogenous and endogenous PML via their RBCC domains. So, for this series of experiments, PML-II- Δ RBCC rather than the full length PML-II was used to test for binding ability of PML-II at the promoters. As demonstrated in Figure 3.2.5 A and B, more ISG15 and ISG56 promoter DNA was precipitated by FLAG antibody from the cells transfected with PML-II- Δ RBCC compared to the empty vector pCI-neo. The specific binding ability of PML-II at promoter was further demonstrated by using another PML isoform, PML-V- Δ RBCC, which had been shown not to bind STAT1. The result showed that PML-V- Δ RBCC did not bind to the promoter of ISG56, in contrast to PML-II- Δ RBCC (Figure 3.2.5 B).

Finally another two pairs of primers that were designed to amplify sequences from promoter or 'far from promoter' regions of a PML target gene were used to test the specificity of PML-II binding to gene promoters. The results showed that more ISG56 promoter DNA was precipitated by FLAG antibody than non-promoter DNA (Figure 3.2.5C). This indicated that PML-II is recruited/bound at the promoter of inducible genes, the expression of which it up-regulates. Taken together, these results provide a further mechanistic understanding of PML-II in the regulation of IFN response.

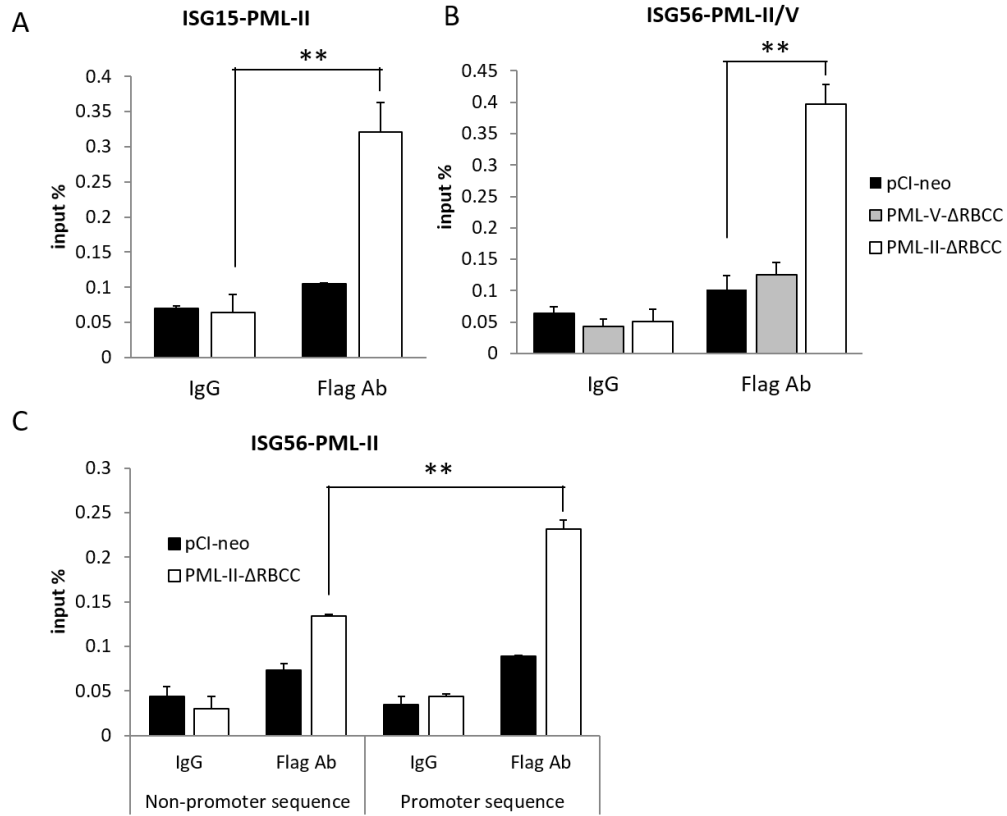


Figure 3.2.5 PML-II binds/is recruited to the promoter of inducible genes. (A) HEK293 cells were transfected with 250 ng/ml Flag-PML-II- Δ RBCC, Flag-PML-V- Δ RBCC, or pCI-neo plasmids, and 48 h later, cells were transfected with poly(I·C) for 16 h. Cell lysates were subjected to ChIP with normal IgG or anti-Flag antibody. Precipitated chromatin DNA was assayed by SYBR green qPCR for (A) ISG15, (B) ISG56 promoter sequences, (C) for both promoter and non-promoter sequences. Results are presented as mean \pm SD of technique triplicate. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

3.3 Discussion

3.3.1 PML-II C-terminal binding

PML currently is known to have seven principal isoforms, which contain an identical N-terminal region and divergent C termini as a result of different RNA splicing. All the principal PML isoforms may have similar function due to the RBCC domain that they share, however, increasing evidence suggests that different PML isoforms have distinct functions mediated by their unique C-terminal domains (Nisole et al, 2013). Bioinformatic analysis showed that the C-terminal region of PML-II was likely to be unstructured but with the propensity to become ordered upon interaction with partner proteins (Leppard et al, 2009). Mutational analysis revealed that one particular molecular recognition element in the C-terminal region of PML-II was required for its interaction with Ad5 E4 Orf3 protein (Leppard et al, 2009). Recently, based on its chemistry and localization properties, the unique C-terminal domain of PML-II has been speculated to interact with transcription factors (Geng et al, 2012). In the present study, PML-II C-terminal sequences were demonstrated to be essential for PML-II binding with transcription factors including NF- κ B and STAT1. Interestingly, the deletion of the PML-II N-terminal RBCC domain did not affect its interaction with TFs. However, deletion of particular sequences, residue 645-684, in the C-terminal of PML-II did reduce its binding ability with these factors. The sequence from 645 to 684 is encoded by exon 7b of the PML-II gene, the part of the protein that makes PML-II distinct from other PML isoforms.

In the Co-IP experiments, the amount of PML-II-RBCC- Δ 1 that was expressed and/or precipitated by Flag antibody was reduced compared with other PML-II mutants. The amount of PML-II-RBCC- Δ 1 was similar to that of other mutants when expressed in unstimulated cells but upon stimulation with poly(I:C), the amount of PML-II-RBCC- Δ 1 was reduced greatly. This instability of the PML-II-RBCC- Δ 1 protein is probably because poly(I:C) stimulation activates the expression of other genes such as ISG15, which has the potential to trigger the degradation of target proteins (Zhou et al, 2017). This result also suggests that C-

terminal $\Delta 1$ region of PML-II is not only important for PML-II binding with TFs but also is essential for PML-II structure and stability. In contrast to the $\Delta 1$ protein, the PML-II- Δ RBCC- $\Delta 2$ mutant protein was very stable in both cases. However, deletion of $\Delta 2$ from PML-II- Δ RBCC impaired PML-II binding ability to NF- κ B and STAT1, while removal of the regions defined by the Δ RBCC and $\Delta 3$ deletions had no effect on their binding ability.

3.3.2 PML-II C-terminal function

It was recently reported that the unique C-terminal domain of PML-II can bind to PML-NBs independent of the shared N-terminal region (Geng et al, 2012). This finding suggested that C-terminal PML-II might be able to replace full-length PML-II for some functions. In this context, it was found that deletion of the N-terminal 360 amino acids of PML-II further enhanced the expression of IFN β and ISGs while full-length PML-II only modestly increase the expression of these genes. This suggested that the N-terminal RBCC region may have negative effects on some functions of PML protein, perhaps contributing to keep some balance *in vivo*. Potentially therefore, some other PML isoforms may also have the potential to activate more potently some other classes of gene expression if they lost the N-terminal regions. Full length PML-IV is already known to activate p53-dependent gene expression (Bischof et al, 2002). Removal of the RBCC domain gave a PML-II protein with enhanced activity in the expression of IFN β and ISGs; the biological significance and mechanism of this effect remains to be determined, but it could reflect a greater functional availability of the PML-II C-terminal domain when less tightly tethered to PML-NB. Taken together, these results provided further evidence of PML-II, particularly its distinct C-terminal domain, playing a key role in the regulation of IFN responses.

3.3.3 PML-II binding at promoters

Results from the current study have shown that PML-II can bind at a gene promoter in response to stimulation. This provided strong evidence that PML-II participates in the gene regulation. PML-II was recruited to the promoter of

activated ISGs most likely is because of its association with various transcription factors or co-activators that are recruited there, as another isoform, PML-V, which does not bind these transcription factors, was not recruited to these promoters. However, we cannot exclude the possibility that PML-II associates with some other transcription-related factors, such as the general transcription factors, which may also contribute to its binding to these promoters.

Collectively, the data in this chapter have shown that specific sequences in the unique PML-II C-terminal domain are essential for its interaction with transcription factors such as NF- κ B and STAT1, while the N-terminal RBCC domain is dispensable (Figure 3.3.1). The recruitment of PML-II at active gene promoters suggests a possibility that PML-II recruited in this way regulates the basal/general transcriptional mechanism, perhaps including histone DNA modification and other chromatin remodeling protein such as SWI/SNF complex assembly or function in gene transcription. This is investigated in the following chapter.

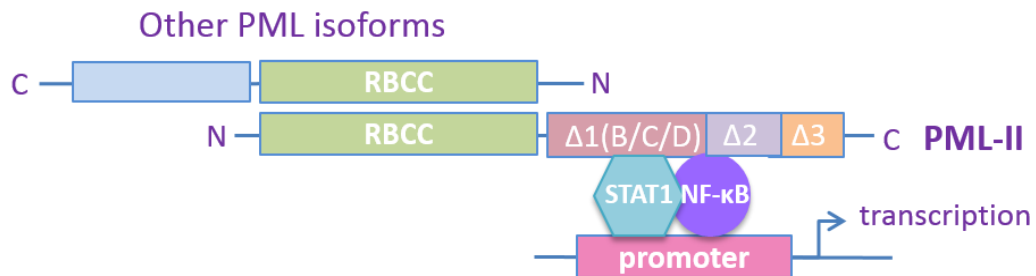


Figure 3.3.1 Proposed model of PML-II C-terminal function in gene transcription

**Chapter 4 PML and chromatin remodeling
protein Brg-1 collaboratively regulate gene
transcription**

4.1 Introduction

The modification of chromatin structure is increasingly recognized to be an important facet for transcriptional regulation. Upon transcriptional activation, the highly ordered structure of the chromatin is modified by the recruitment of various histone-modifiers and chromatin remodeling complexes and finally forms an opening chromatin structure for the binding of activators and members of the general transcription machinery.

The SWI/SNF enzymatic complexes are large multimeric assemblies which are thought to be recruited to specific gene targets through association with DNA-binding transcription factors, co-regulators, or members of the general transcriptional machinery. In addition to ATP-dependent chromatin modifiers, various modifications on histone tails also play an important role in regulating chromatin structure and gene transcription (Kouzarides, 2007). Histone modifications within target gene promoters may serve as an interaction surface for the assembly and /or recruitment of co-regulator complexes such as SWI/SNF to the DNA and eventually regulate the expression status of these genes.

SWI/SNF complexes have been shown to regulate expression of multiple interferon-responsive genes (Agalioti et al, 2000; Huang et al, 2002). For example, Brg-1 is involved in IFN β transcription (Agalioti et al, 2000). Brg-1 is also required for the expression of MHC class II and guanylate-binding protein 1 (GBP1) as the induction of both genes by IFN- γ is defective in a Brg-1/BRM-deficient cell line (SW13) (Pattenden et al, 2002). Mechanistically, the absence of Brg-1 blocks the recruitment of transcription factor STAT1 to the CIITA locus so reducing its expression (Ni et al, 2008; Pattenden et al, 2002). Moreover, depletion of Brg-1 impeded ISGF3 (STAT1/STAT2/IRF9) complex binding to several ISGs, such as GBP1, IFI27 and IFITM1 promoter in response to IFN α stimulation, resulting in impaired gene expression (Ni et al, 2005).

Recent work in our laboratory had demonstrated that PML-II plays an important regulatory function/role in the induced transcription during IFN and inflammatory responses (Chen et al, 2015). Depletion of PML-II impaired specific transcription factor and coactivator CBP recruitment to the promoters of

inducible genes (Chen et al, 2015). This suggested that PML-II may be a master regulator with a general regulation function in gene transcription. To address this hypothesis, the purpose of this chapter was to dissect PML-II regulation function on the basal transcriptional machinery, including chromatin remodelling and histone modification, and the link between PML-II and Brg-1 in this process.

4.2 Results

4.2.1 Brg-1 can be knocked down by siRNA

To establish an experimental system in which Brg-1 could be studied, the knockdown efficiency of Brg-1 by siRNA was tested. As shown in Figure 4.2.1, Brg-1 protein levels were decreased by Brg-1 siRNA in a concentration dependent pattern, the expression of Brg-1 being greatly inhibited at the concentration of 125 pmol/ml siRNA compared to the control siRNA (Figure 4.2.1A). The efficiency of 125 pmol/ml Brg-1 siRNA knockdown was further confirmed by testing Brg-1 mRNA levels (Figure 4.2.1B). 125 pmol/ml siRNA therefore was used as the optimized concentration for Brg-1 knockdown in the following experiments.

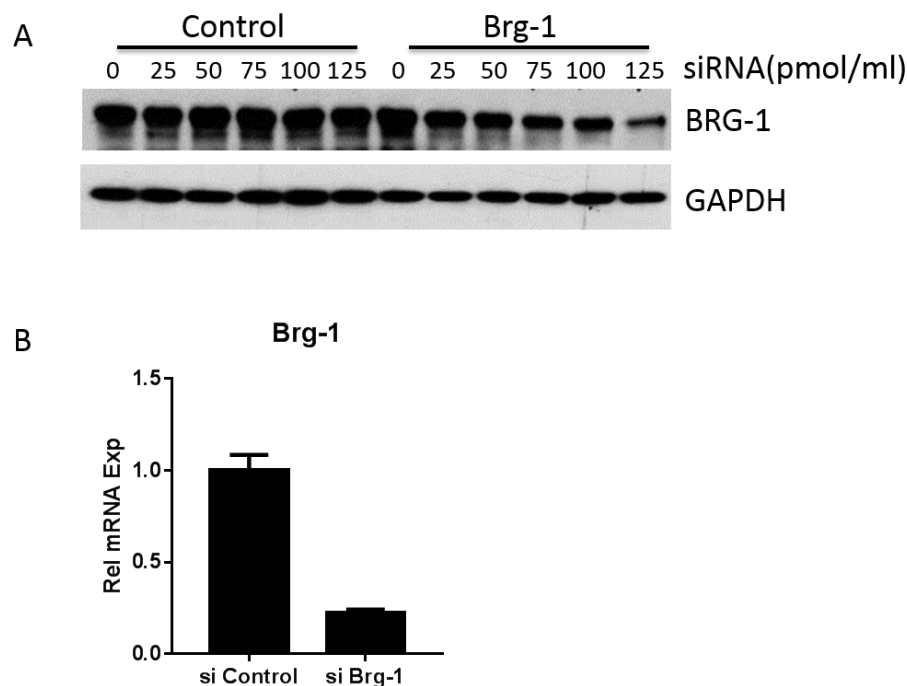


Figure 4.2.1 Brg-1 can be knocked down by siRNA. (A) HEK293 cells were transfected with 0, 25, 50, 75, 100, 125 pmol/ml siBrg-1 or siControl and incubated for 48 h, then the cells were harvested. Cells lysates were separated by 8% SDS-PAGE and subjected to western blotting for either Brg-1 or GAPDH (loading control). (B). HEK293 cells were transfected 125 pmol/ml control or Brg-1 siRNA for 48 h, and then total RNA was extracted and Brg-1 mRNA quantified by SYBR-Green RT-qPCR. The data shown are relative to control siRNA and are the mean \pm standard deviation of three replicates

4.2.2 Knockdown of Brg-1 or PML-II downregulates the expression of ISGs

Previous studies showed that Brg-1 is required for the expression of a group of ISGs (Agalioti et al, 2000; Ni et al, 2005; Pattenden et al, 2002), and recent work in our laboratory demonstrated that PML-II is also important for regulating ISGs expression (Chen et al, 2015). To study the relationship between PML-II and SWI/SNF chromatin remodeling protein Brg-1, the effect of knockdown of either Brg-1 or PML-II was tested on the expression of a subset of ISGs such as GBP1, IFI27, ISG54, ISG15 and ISG56 in HeLa cells. The results showed that the

mRNA expression of GBP1, IFI27, ISG15 and ISG54 was severely decreased by depleting Brg-1, while the knockdown of Brg-1 only modestly reduced the expression of ISG56. This suggested that the relative dependence on Brg-1 may vary between different genes (Figure 4.2.2). Consistent with our previous reports, knockdown of PML-II severely affected the expression of all these genes tested, confirming that PML-II regulates IFN and ISGs gene transcription (Figure 4.2.2). This downregulation of induced gene expression by depleting Brg-1 and PML-II was also observed in HEK293 cells (Figure 4.2.6). Together, these results confirmed the notion that both Brg-1 and PML-II are essential in the induction of ISG transcription.

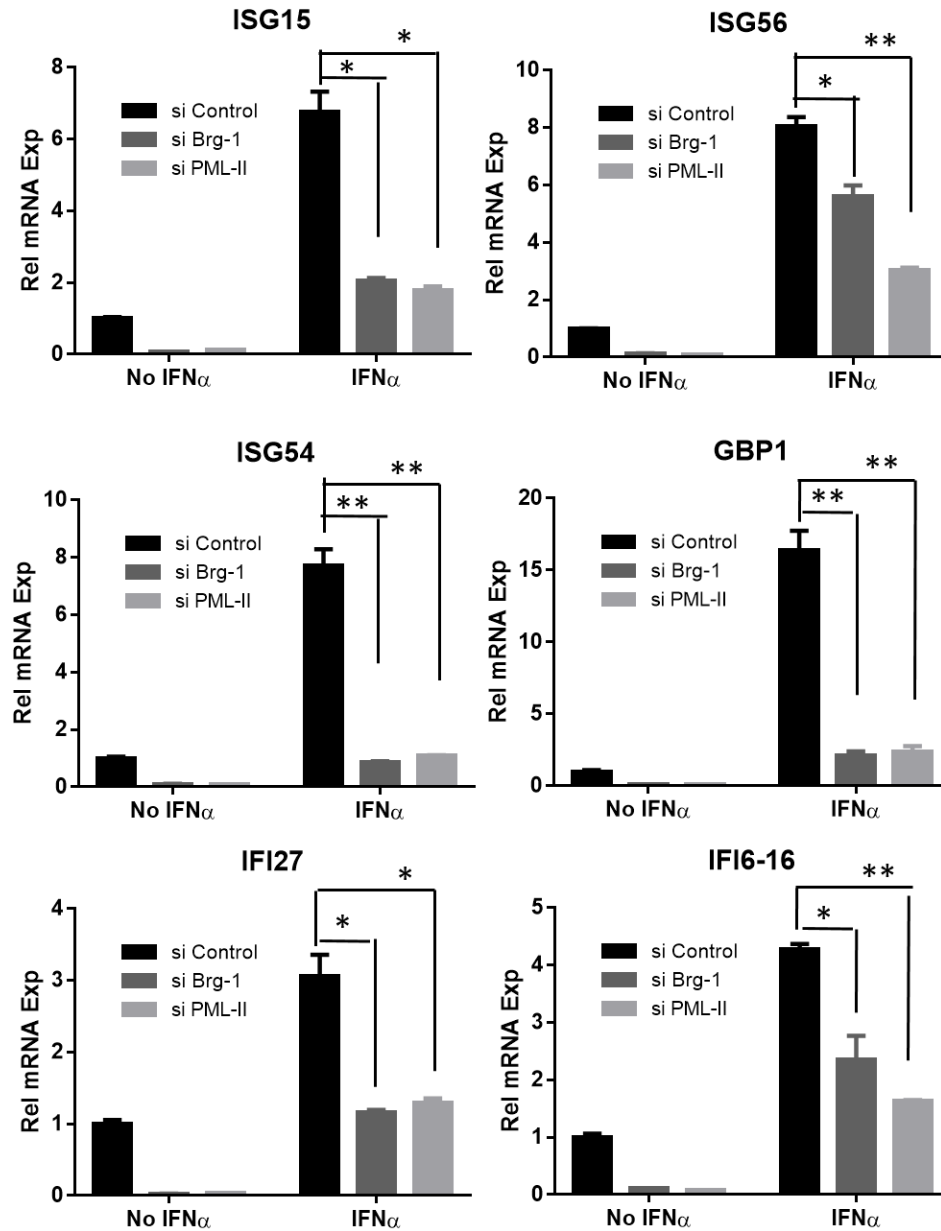


Figure 4.2.2 Knockdown of Brg-1 or PML-II downregulates the expression of ISGs. HeLa cells were treated with 125 pmol/ml PML-II, Brg-1 or Control siRNA for 48 h, and stimulated with IFN α for 16 h. mRNA expression levels were measured by SYBR Green qPCR. The values presented are relative to control siRNA with no-stimulation and are the mean \pm standard deviation of three technical replicates. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

4.2.3 Time course of dynamic chromatin modification at ISG promoters

Histone modifications are thought to cooperate with chromatin remodeling complexes to reconfigure chromatin, thereby establishing a local chromatin structure and facilitating the subsequent assembly of an active preinitiation complex at the promoter (Aaronson & Horvath, 2002; Reith & Mach, 2001). The levels of H3K4me3 and H3K9me3 function as marks of gene transcription activation and silencing, respectively. Given Brg-1 and PML-II are required for gene transcription, and the depletion of PML-II or Brg-1 inhibits gene transcription, it was asked whether knockdown of PML-II and Brg-1 affects histone modification at interferon response promoters. The dynamic change of H3K9me3 and H3K4me3 histone modification at selected promoters in response to gene activation was tested firstly. The relative ChIP signal of each gene target in a recovered ChIP DNA sample was expressed as % input specifically precipitated, with input DNA being quantified in parallel from DNA purified from a sample of chromatin reserved at the nuclear lysate stage. As observed in Figure 4.2.3, the basal level of H3K4me3 at ISG54 and ISG56 promoter was significantly higher than that of H3K9me3 in inactive cells. However, upon IFN α stimulation, the level of H3K4me3 at the ISG54 gene promoter was increased greatly to (7-fold) after 8 hours IFN α treatment. Similarly, in the promoter of ISG56, the enrichment of H3K4me3 reached a peak at 8h after stimulation, a level nearly three-fold more than basal level (non-stimulation) (Figure 4.2.3).

However, the level of H3K9me3 at ISG54 promoter was decreased immediately after IFN α treatment, and the downward trend continued for the initial 6h then followed by recovery to the basal level (see Figure 4.2.3). Similarly, over the same period the level of H3K9me3 in the ISG56 promoter was suppressed to a relatively low level in response to IFN α stimulation, then showed some increase at 8 hour, but its levels was still lower than the basal level (Figure 4.2.3).

The time course of CBP binding at these promoters during stimulation was tested since it is a very important histone acetyltransferase (HAT) (Bannister & Kouzarides, 1996). Interestingly, the temporal pattern of CBP recruitment is very

similar to that of H3K4me3 modification although the signals were generally weak (Figure 4.2.3).

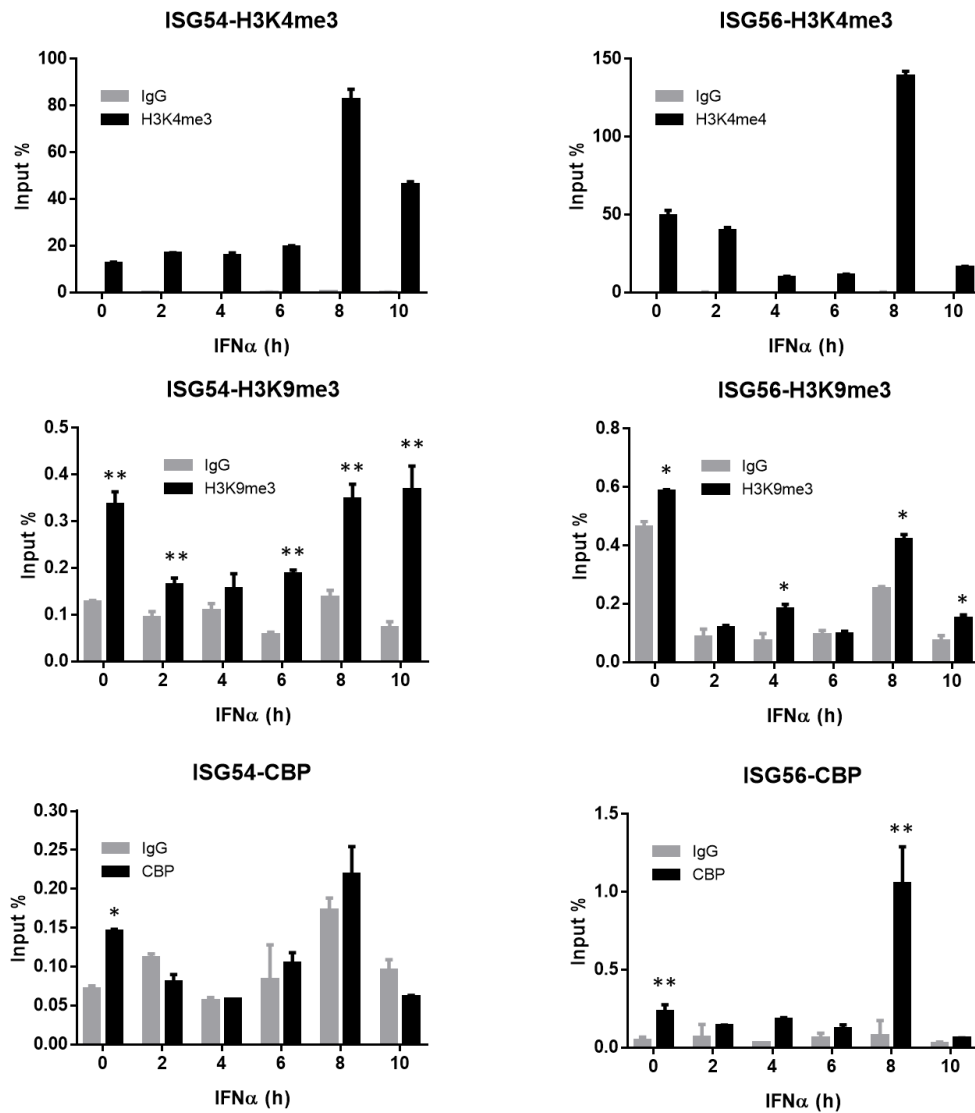


Figure 4.2.3 Time course of dynamic chromatin modification at ISG promoters. 95% confluent Hela cells were stimulated with IFNα for 0, 2, 4, 6, 8, 10h and then cells were collected and equal aliquots of fixed chromatin were subjected to ChIP precipitation with normal IgG, H3K4me3, H3K9me3 and CBP antibody respectively. Precipitated chromatin DNA was assayed by SYBR green qPCR for ISG54, ISG56 promoter sequences. Results are presented as mean ± SD of technical triplicate. One tailed student t-test; * p<0.05, ** p<0.01. All signals in the H3K4me3 graphs were highly significant.

4.2.4 Depletion of either Brg-1 or PML-II affects histone modification

Having tested the dynamics of H3K9me3 and H3K4me3 enrichment/disposition at gene promoter (Figure 4.2.3), the effect of knockdown of PML-II or Brg-1 on H3K9me3 and H3K4me3 at gene promoters was tested. As both PML-II and Brg-1 are positive regulators for ISG transcription, the depletion of either PML-II or Brg-1 might increase or maintain level of H3K9me3, and/or decrease H3K4me3 level at the promoter. As expected, the level of H3K9me3 was decreased in response to IFN α stimulation but the knockdown of either PML-II or Brg-1 increased/recovered the level of H3K9me3 at these promoters (Figure 4.2.4 A). This is consistent with a recent study where silencing of PML enhanced the H3K9me3 level at the Oct4 gene promoter in P19 embryonal carcinoma (EC) cells (Chuang et al, 2011). Surprisingly, the level of H3K4me3 was not decreased but slightly increased by depleting PML-II or Brg-1 (Figure 4.2.4 B).

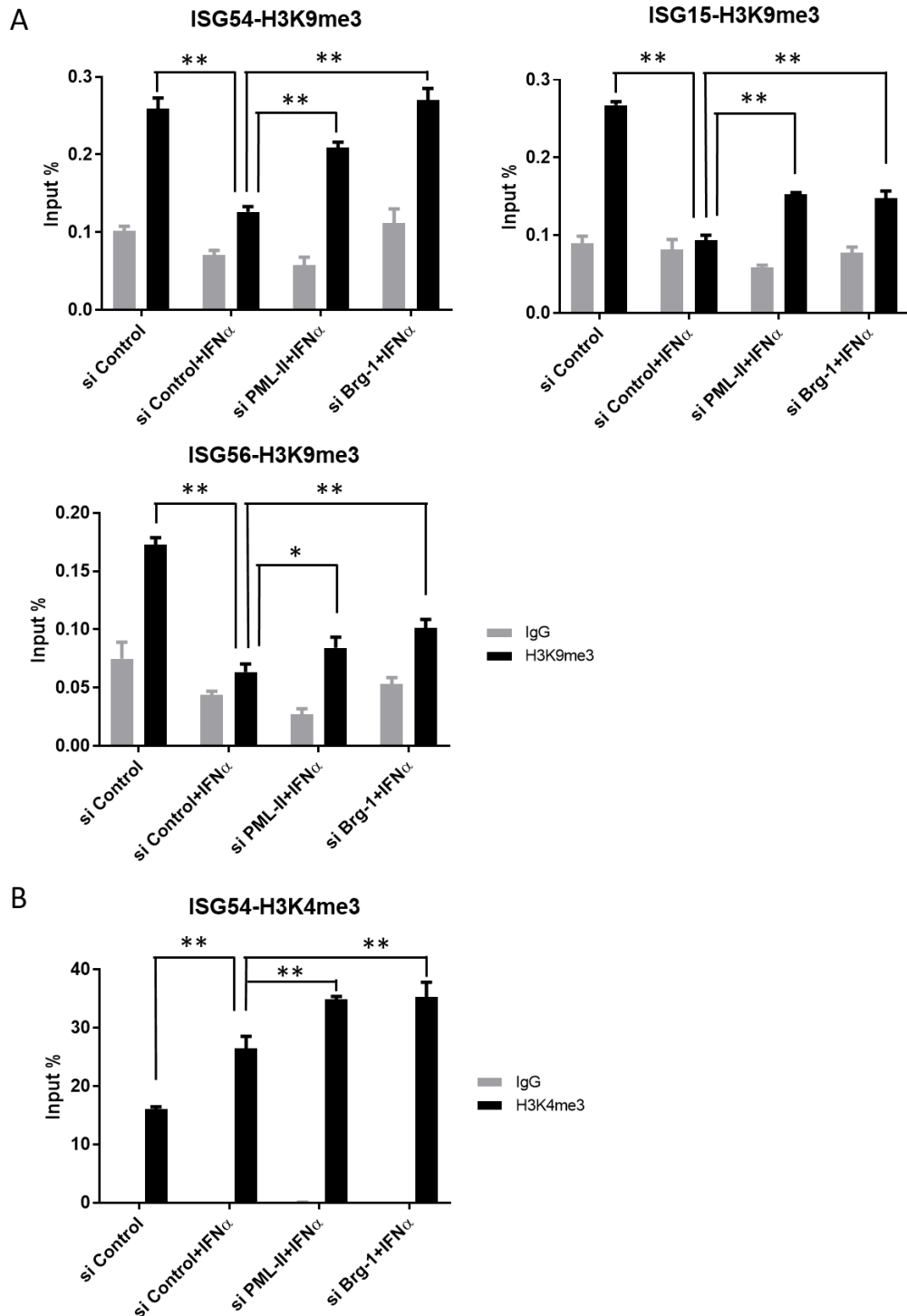


Figure 4.2.4 Depletion of either Brg-1 or PML-II affects histone modification at ISGs. (A) HeLa cells were transfected with 125pmol/ml control, PML-II or Brg-1 siRNA for 48 h, then treated with 1000 unit/ml IFNα for 6 h (H3K9me3) or (B) HeLa cells were transfected with siRNA as A and stimulated with 1000 unit/ml IFNα for 8 h (H3K4me3), and then cells were collected and equal aliquots of fixed chromatin were subjected to ChIP precipitation with normal IgG, H3K4me3 or H3K9me3 antibody respectively. Precipitated chromatin DNA was assayed by SYBR green qPCR for ISG15, ISG54 and ISG56 promoter sequences. Results are presented as mean \pm SD of technical triplicate. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

4.2.5 Loss of either Brg-1 or PML-II affects transcription factor STAT1 recruitment at ISG promoters

Other studies and our previous work (Chen et al, 2015; Ni et al, 2005) have shown knockdown of Brg-1 or PML-II affects transcription factor STAT1 binding at ISG promoters. To confirm and extend this, transcription factor binding at these promoters was tested. Firstly, the binding time-course of STAT1 to the ISG15/54/56 genes in response to IFN α stimulation was tested (Figure 4.2.5A). The result showed that upon stimulation the binding of STAT1 at the promoter was gradually increased and reached the highest level at 4-6 hours after stimulation. This peak of STAT1 binding was followed by a slow dropdown but the level was still higher than basal level. This result showed that the recruitment of transcription factor STAT1 to these promoters is an earlier event than the recruitment of CBP or the changes in histone modification just described (Figure 4.2.3).

Next, the effect of depletion of Brg-1 and PML-II on the binding of STAT1 at these promoters was detected. The level of STAT1 binding was decreased in the absence of either Brg-1 or PML-II (Figure 4.4B). It is notable that the level of STAT1 reduction caused by depleting PML-II or Brg-1 is very similar, confirming the function of PML-II in gene transcription and suggesting a potential interaction of PML-II with Brg-1 in this process. Together, this result is consistent with others' and our previous reports (Chen et al, 2015; Ni et al, 2005) and providing support for the idea that both Brg-1 and PML-II are required for STAT1 binding at ISG promoters.

Collectively, the effects of PML-II and Brg-1 removal on histone modification and transcription factor STAT1 binding provide a mechanistic hypothesis for their regulatory function in gene transcription and also further evidence that Brg-1 and PML-II play an important role in interferon response gene transcription.

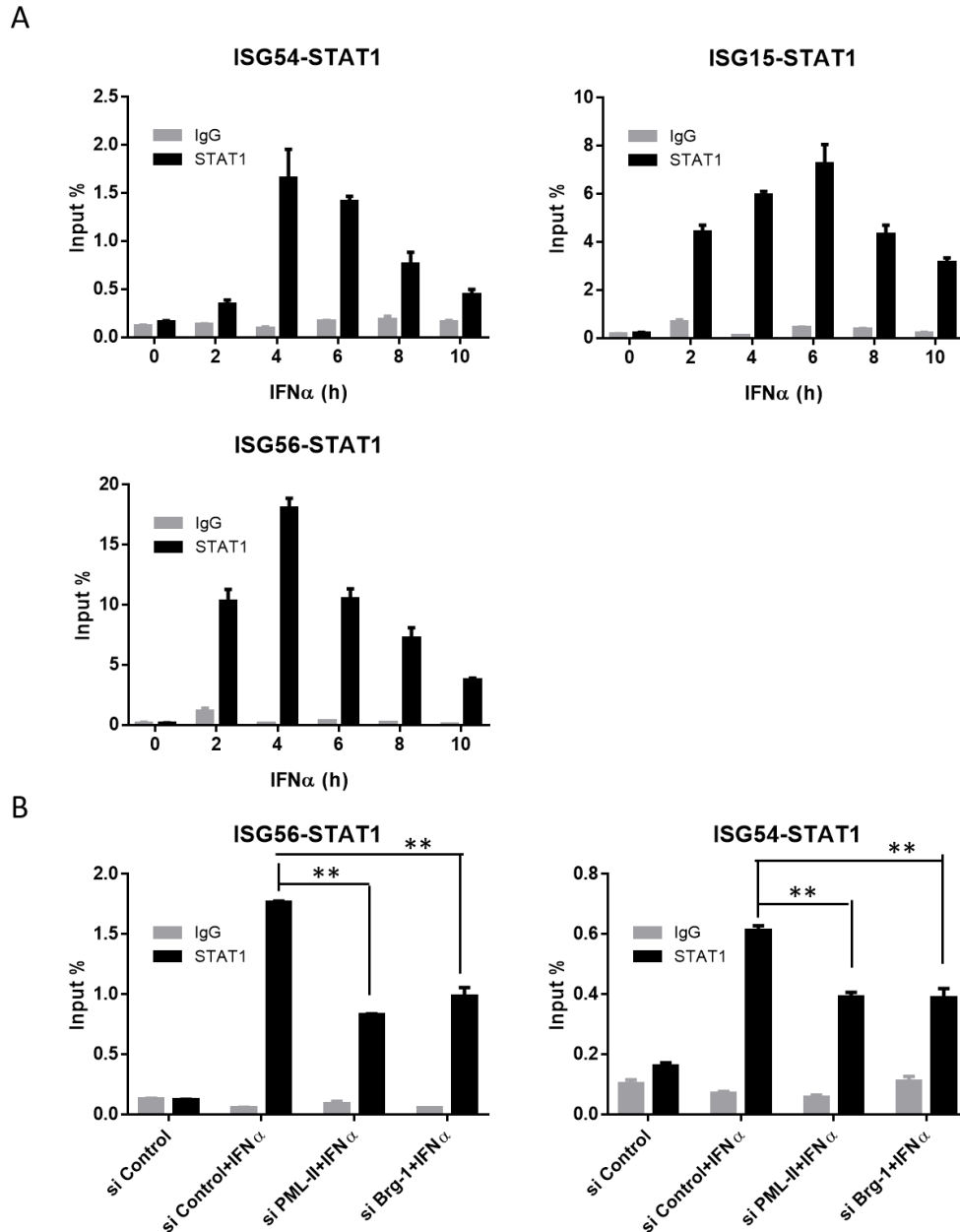


Figure 4.2.5 Losing either Brg-1 or PML-II affects transcription factor STAT1 recruitment at ISG promoters. (A) HeLa cells were treated with 1000 unit/ml IFN α for the times indicated. (B) HeLa cells were transfected with 125 pmol/ml control, PML-II or Brg-1 si RNA for 48 h, then treated with 1000 unit/ml IFN α for 6 h. Cells were then collected and equal aliquots of fixed chromatin were subjected to ChIP precipitation with normal IgG and STAT1 antibody. Precipitated chromatin DNA was assayed by SYBR green qPCR for ISG54, ISG56 and ISG15 promoter sequences. Results are presented as mean \pm SD of technical triplicate. STAT1 binding during IFN stimulation was significantly ($P < 0.05$) or highly significantly ($p < 0.01$) increased over the $t = 0$ value at all time points by Student t-test, panel A. The reduction in binding after specific PML or Brg-1 siRNA treatment was highly significant by the same test as indicated in B.

4.2.6 PML-II and Brg-1 cooperatively regulate Brg-1-dependent gene expression

Having demonstrated that both PML-II and Brg-1 regulate ISGs transcription, it was asked next whether double knockdown of PML-II and Brg-1 would further enhance the inhibition effect on gene transcription, greater than knockdown of either one (Figure 4.5). As before, knockdown of either Brg-1 or PML-II alone significantly reduced the mRNA expression of a group of genes including IFN β , ISG54, IFI27, ISG15 and GBP1, while the expression of ISG56 was less affected by depleting Brg-1. Depletion of both PML-II and Brg-1 further decreased the mRNA level of IFN β , ISG54, IFI27 and GBP1 genes. This suggested that PML may play a role in Brg-1-containing transcriptional complex assembly or mediated-modification, or may collaborate with Brg-1 in gene transcription. However, an additive effect of double knockdown of Brg-1 and PML-II on the expression of ISG15 and ISG56 was not observed (Figure 4.2.6). These data may suggest the expression of these genes is less dependent on Brg-1, or the functions of PML-II and Brg-1 in this gene transcription are overlapping/redundant so that the double knockdown could not further inhibit the expression of these genes.

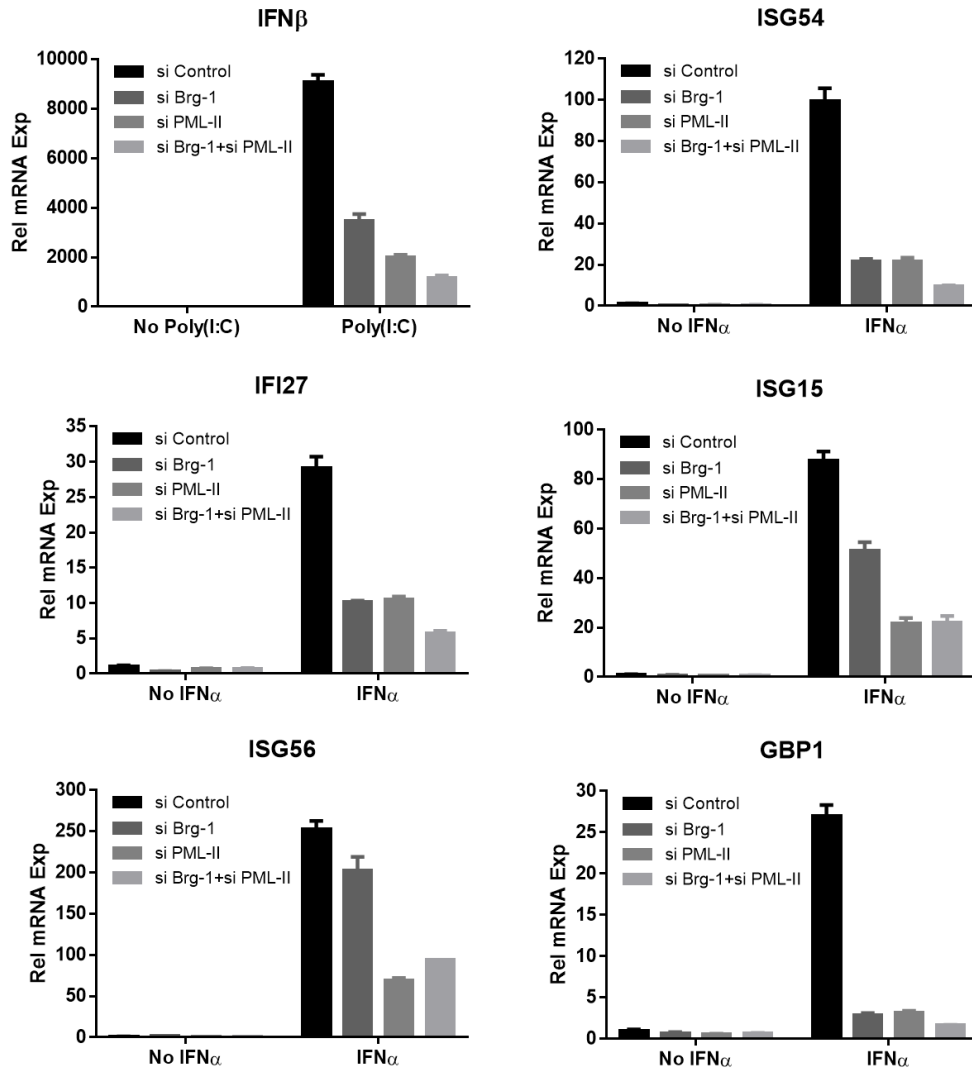


Figure 4.2.6 PML-II and Brg-1 cooperatively regulate Brg-1-dependent gene expression. HEK293 cells were treated with 125 pmol/ml PML-II, Brg-1, Control or both PML-II and Brg-1 siRNA for 48 h, and stimulated with poly(I:C) or IFN α for 16 h. mRNA expression levels were measured by SYBR Green qPCR. The values presented are relative to control siRNA with no-stimulation and are the mean \pm standard deviation of three technical replicates. Reductions in IFN-induced gene expression following specific siRNA treatment were all significant or highly significant by Student t-test.

4.2.7 PML-II is physically associated with Brg-1

To investigate the possible regulation mechanism of PML-II on Brg-1-containing SWI/SNF transcription complexes, A co-immunoprecipitation (co-IP)

experiments to test the interaction of PML-II and Brg-1 was carried out. To do this, Hela cells were transfected with a plasmid expressing Flag-tagged full-length PML-II, PML-V or pCI-neo (empty vector control) and carried out immunoprecipitation experiments with anti-Flag-beads. Full-length PML-II could co-precipitate Brg-1 (Figure 4.2.7A). Strikingly though, the full-length PML-V isoform could also form a protein complex with Brg-1 (Figure 4.2.7A). Potentially, therefore both isoforms might interact with Brg-1 complexes via their distinct C-terminal domains. Alternatively, this binding to Brg1 might be mediated by the N-terminal RBCC structure found in all PML isoforms, or it might be that the binding to Brg-1 by PML-V is an indirect event, mediated through the association of the tagged PML-V with endogenous PML isoforms possess direct binding ability for Brg-1 (e.g. PML-II), because all PML isoforms can bind to each other through their RBCC structures.

To rule out/exclude the possibility that PML-V binding to Brg-1 is an indirect binding through the association with other PML isoforms, cells were transfected with plasmid expressing N-terminal RBCC deleted/truncated PML-II and PML-V, named PML-II- Δ RBCC and PML-V- Δ RBCC. PML-II- Δ RBCC had a strong ability to bind Brg-1 even though its transfection efficiency was lower compared to PML-V- Δ RBCC, while PML-V- Δ RBCC only showed a very weak association with Brg-1 (Figure 4.2.7 B). Interestingly, PML-II- Δ RBCC also specifically bound to transcription coactivator CBP while PML-V- Δ RBCC failed to do this (Figure 4.2.7 B). Thus Brg-1 associates specifically with the PML-II C-terminal domain.

The binding of endogenous PML-II with Brg-1 was also tested (Figure 4.2.7 C). In this experiment, both Brg-1 and PML-II antibodies were used for precipitation to test for interaction in both directions. As expected, Brg-1 antibody could precipitate PML-II protein. However, the reciprocal test could not detect this interaction as no Brg-1 was detected when PML-II antibody was used for the precipitation. This perhaps is because the specific PML-II antibody binding site in PML-II protein is occupied by Brg-1 so PML-II antibody could not pull down Brg-1; the test with exogenous expressed PML-II works because that used Flag antibody to precipitate rather than an internal PML epitope. The association of

both Brg-1 and PML-II with STAT1 was also observed, which is consistent with previous reports (Chen et al, 2015; Zhang et al, 2010). These results have shown that endogenous PML-II as well as ectopically expressed PML-II can physically interact with BRG1. Taken together, these data indicate that PML-II can interact with Brg-1 suggesting a possibility that the interaction regulates Brg1 functions in gene transcription.

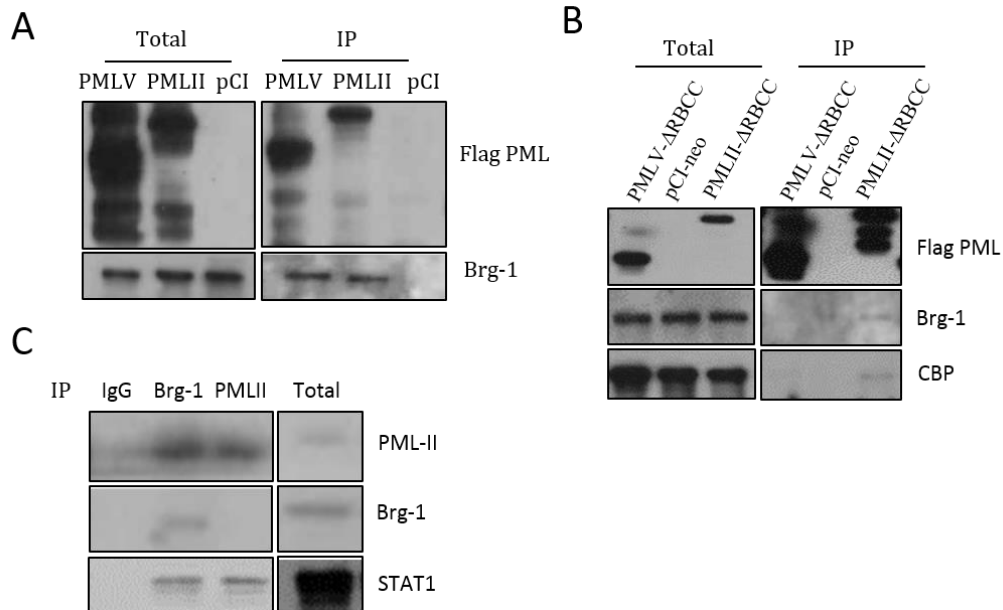


Figure 4.2.7 PML-II is physically associated with Brg-1. (A) HeLa cells were transfected with 250 ng/ml Flag-PML-II, Flag-PML-V plasmids or pCI-neo empty vector for 24 h, (B) HeLa cells were transfected with 250 ng/ml Flag-PML-II-ΔRBCC, Flag-PML-V-ΔRBCC or pCI-neo empty vector for 24 h, then stimulated with poly(I:C) for 10 h, lysates prepared, immunoprecipitated with anti-Flag beads and precipitates & total lysates analysed for Flag-PML, Brg-1 or CBP or STAT1 by western blotting. (C) Confluent HeLa cell were stimulated with poly(I:C) for 10 h, lysates prepared and aliquots immunoprecipitated with IgG, Brg-1 or PML-II antibody. Precipitates and total lysates were then analysed for PML-II, Brg-1 or STAT1 by western blotting.

4.2.8 PML-II C-terminal Δ D fragment is essential for PML-II associated with Brg-1 and CBP

In order to further map the functional fragments of PML-II which are responsible for binding to Brg-1, flag-tagged full length PML-II, PML-II Δ RBCC and a pre-existing set of mutants which are further deleted in specific sequences from the C terminal domain (Leppard et al, 2009) were used to test their association with Brg-1. As is seen in Figure 4.2.8A, each of the PML mutants tested was efficiently expressed and could be precipitated by anti-Flag agarose beads, however the amount of Brg-1 co-precipitated with the various Flag-PMLs varied greatly indicating that some of the mutations had affected sequences necessary for Brg-1 binding. PML-II and II Δ RBCC co-precipitated significant amounts of Brg-1 (Figure 4.2.8B). However, Δ m1 Δ m2 mutant was unable to co-precipitate Brg-1. The amount of Brg-1 co-precipitated was also greatly affected by mutation Δ B, Δ 7 and Δ D. These results therefore show that PML-II residues 645–674, comprising the sequence deleted in Δ m1 plus Δ 7, are required for Brg-1 binding (Figure 4.2.8B). The binding of this panel of PML-II mutants with CBP was also tested, which can bind to all the PML isoforms through an interaction with sequences in the common N terminal domain. Interestingly, it was found that Δ D also disrupted sequences essential for PML-II binding with CBP, as very little CBP was precipitated from cells transfected by Δ D plasmid (Figure 4.2.8B). The CBP binding ability was also impaired with various degrees in the Δ m1 Δ m2, Δ 7 and Δ B PML-II mutants (Figure 4.2.8B). This suggested the PML-II sequence required for Brg-1 binding is similar to that required for CBP binding.

The specific association of PML-II with Brg-1 widens the regulation function of gene transcription by PML-II. Based on the results above, it can be hypothesised that PML-II regulates Brg-1 function by three possibilities: (1) directly regulating Brg-1 expression; (2) affecting Brg-1-containing complex formation; or (3) affecting Brg-1 recruitment at promoters. These ideas were tested in subsequent experiments.

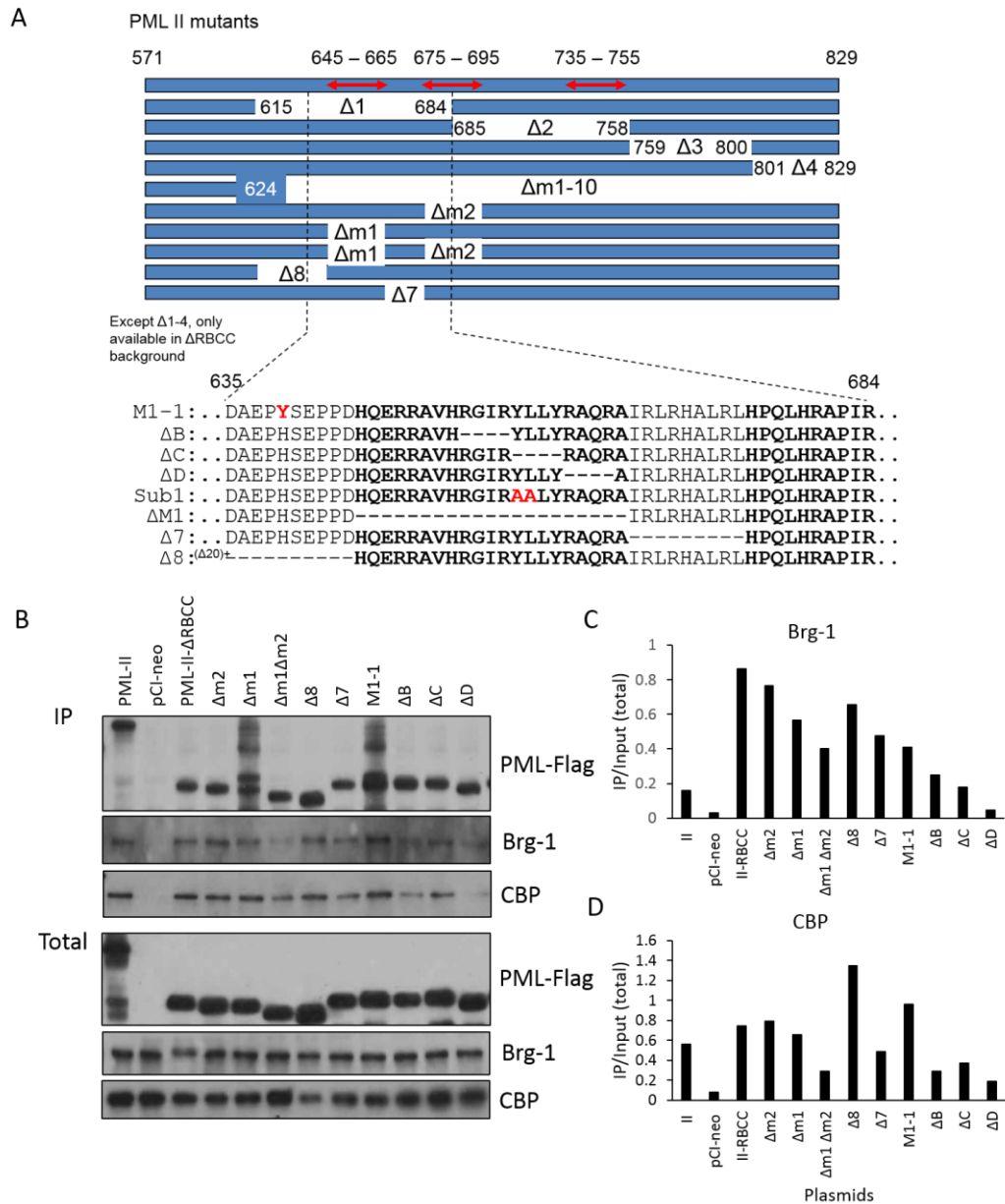


Figure 4.2.8 PML-II C-terminal ΔD fragment is essential for PML-II associated with Brg-1 and CBP. (A) The cartoon represents the unique C-terminal domain that defines PML-II (residues 571 – 829 of full-length PML-II), with the sequences deleted or altered in individual mutants indicated. (B) Hela cells were transfected with 250 ng/ml Flag-PML-II, Flag-PML-II-ΔRBCC, Δm1, Δm2, Δm1Δm2, Δ8, Δ7, M1-1, ΔB, ΔC, ΔD plasmids or pCI-neo empty vector for 24 h, then stimulated with poly(I:C) for 10 h, lysates prepared, immunoprecipitated with anti-Flag beads and precipitates & total lysates analysed for Flag-PML, CBP and Brg-1 by western blotting. (C, D) The precipitated protein bands of Brg-1 and CBP in (B) were quantified by image analysis using QuantityOne software (Biorad) and normalized to total expressed proteins.

4.2.9 Knockdown of PML-II affects expression of some SWI/SNF subunits

Firstly, the effect of knockdown of PML-II on Brg-1 expression was tested. As shown in Figure 4.2.9, the mRNA expression of Brg-1 was slightly increased by PML-II depletion under the conditions tested (Figure 4.2.9A). There was also no obvious decrease in Brg-1 expression at the protein level by depleting PML-II (Figure 4.2.9B). Next, the expression of the other core enzymic subunit of SWI/SNF complex, BRM was also tested. Surprisingly, the mRNA expression of BRM was decreased by knocking down of PML-II (Figure 4.2.9C). This suggested a possibility that the expression of BRM-dependent genes can be regulated by PML-II via an effect on BRM expression.

Although Brg-1 alone can remodel the chromatin, the addition of other core subunits of SWI/SNF complex, such as BAF155 and BAF47, will maximise its function (Euskirchen et al, 2011). PML-II might affect the expression of such non-enzymic components of SWI/SNF, thus affecting the stability or activity of the complex. Therefore, the effect of PML-II knock-down on mRNA levels for representative subunit BAF155 and BAF47 was also tested. The depletion of PML-II reduced BAF47 (Figure 4.2.9D) and BAF155 expression (Figure 4.2.9E) to similar extents. Interestingly, this effect was also similar to the reduction in BRM caused by reducing PML-II. These data suggest an effect of PML-II on Brg-1/or BRM-containing complex formation.

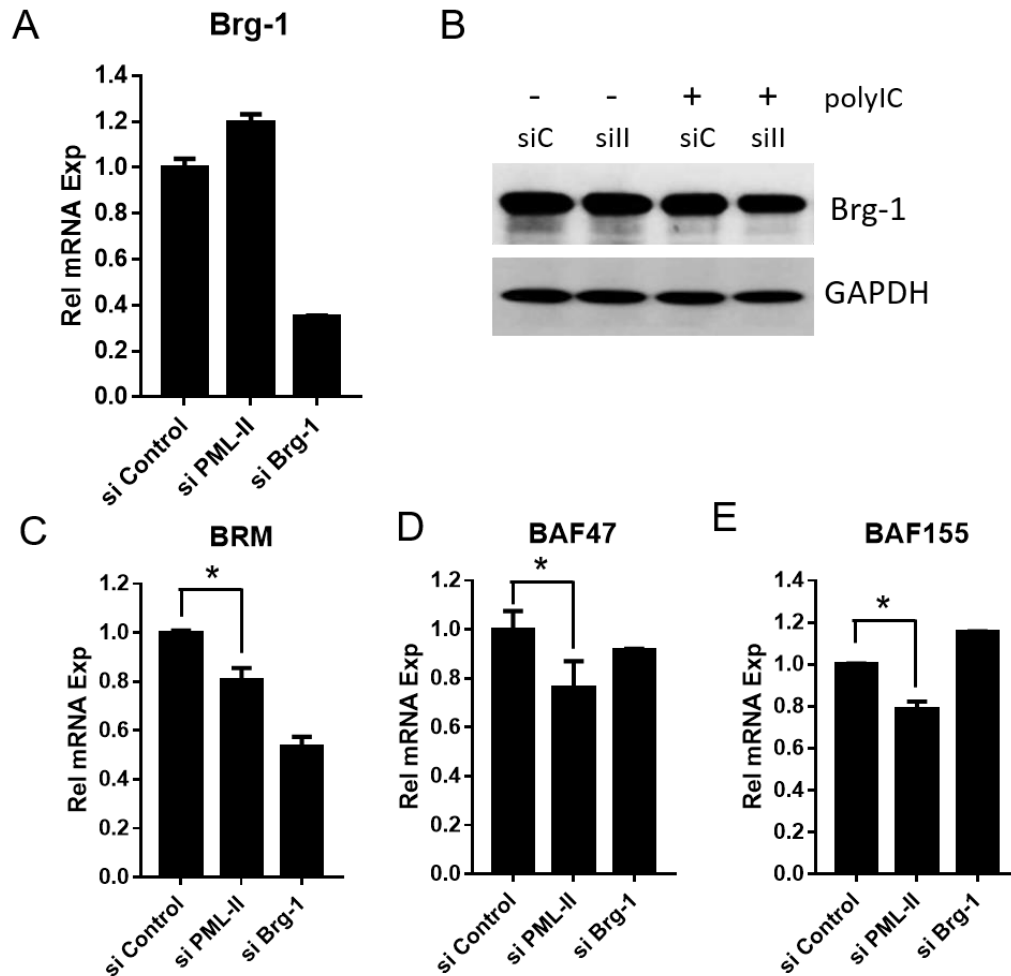


Figure 4.2.9 Knockdown of PML-II affects expression of some SWI/SNF subunits. HEK293 cells were treated with 125 pmol/ml PML-II, Brg-1 or Control siRNA for 48 h, and stimulated with IFN α for 16 h. (A) Brg-1, (C) BRM, (D) BAF47 and (E) BAF155 mRNA expression levels were measured by SYBR Green qPCR. The values presented are relative to control siRNA and are the mean \pm standard deviation of three technical replicates. (B) HEK293 cells were transfected siRNA as A, and stimulated with poly(I:C) for 16 h, and cell lysates were separated by 8% SDS-PAGE and subjected to western blotting for Brg-1 or GAPDH (loading control). Two tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

4.2.10 PML-II, Brg-1 and BAF155 is recruited to the promoter in response to IFN α stimulation

To determine the effect of PML-II on Brg-1 recruitment to promoters, the binding of PML-II and Brg-1 itself at selected promoters was firstly tested by using IFN α stimulation at different time points. Compared to the level of STAT1 and H3K4me3, the basal level of Brg-1 binding at the ISG54 promoters (input %) was much lower, similar to that of H3K9me3 and CBP (Figure 4.2.3 and 4.2.5). IFN α stimulation increased Brg-1 binding somewhat but the level was still not as high as that of STAT1 and H3K4me3 (Figure 4.2.10A). 6 hours after stimulation the level of Brg-1 occupation at ISG54 promoter peaked, which is 2 hours later than the peak binding of STAT1 and 1-2 hours earlier than other factors such as CBP and H3K4me3 (Figure 4.2.10A, 4.2.3 and 4.2.5).

Given the fact that SWI/SNF remodeling complex can have alternative compositions, and BAF155 is one of the most important scaffold/component proteins in both Brg-1-containing and BRM-containing complexes, the kinetic binding of BAF155 at ISG54 and ISG56 gene promoters were tested. The level of BAF155 binding was higher than for Brg-1. The binding pattern of BAF155 was very similar as that of Brg-1 but reached the highest level at 8 hours after stimulation then decreased after that (Figure 4.2.10 B and C).

Given the interaction of PML-II with Brg-1 demonstrated earlier, the binding of PML-II to ISG promoters was also tested. IFN α stimulation increased endogenous PML-II recruitment to ISG54 promoter and reached the highest level at about 8-10 hours after stimulation, similar to CBP, H3K4me3 and BAF155, 2 hours later than Brg-1 recruitment and 4 hours later than STAT1 DNA binding. As this PML-II signal was weak, the recruitment of exogenously expressed Flag-PML-II was tested, which showed a similar temporal pattern with a clearer signal (Figure 4.2.10 E).

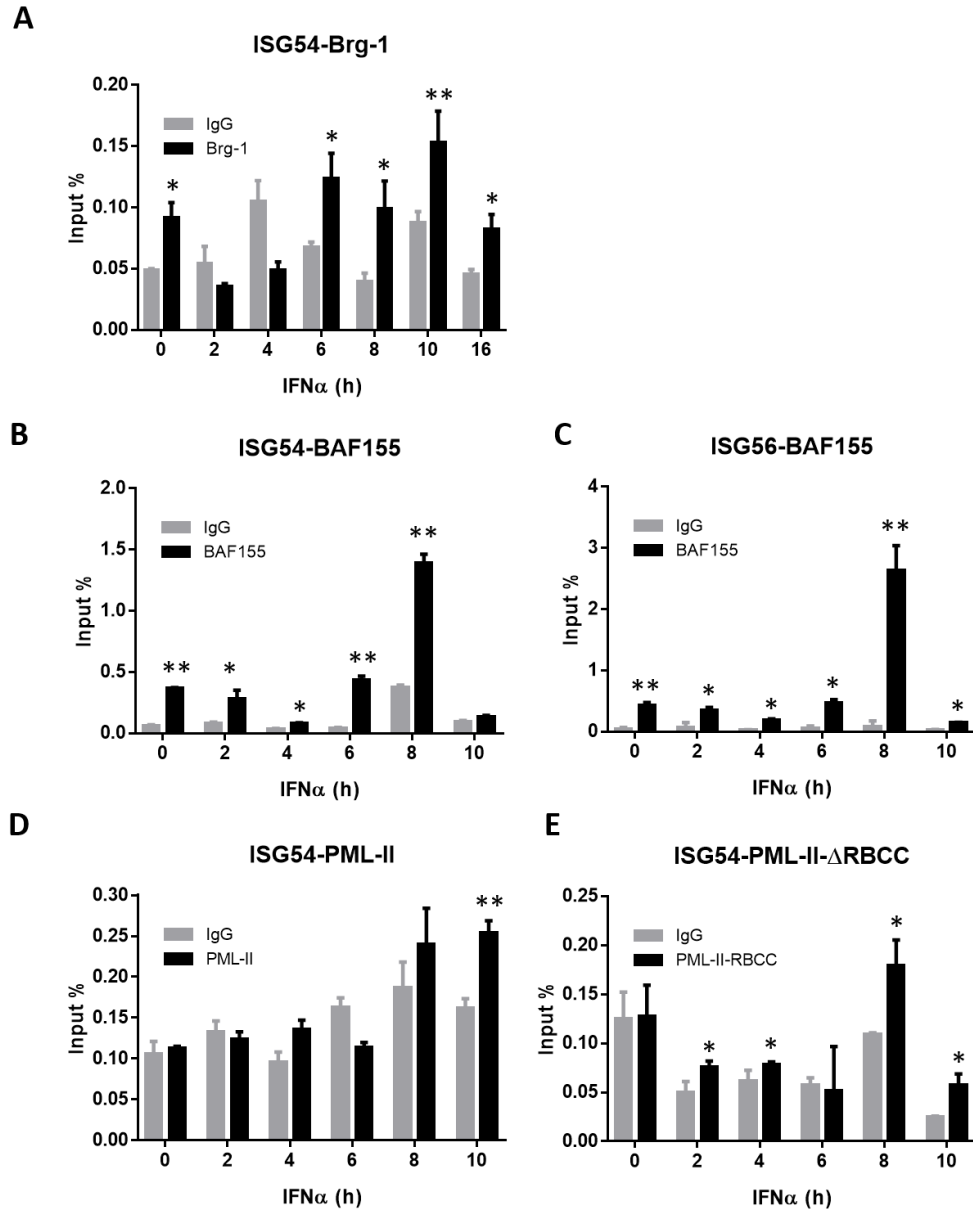


Figure 4.2.10 PML-II, Brg-1 and BAF155 are recruited to promoters in response to IFN α stimulation. Confluent Hela cells were stimulated with IFN α for 0, 2, 4, 6, 8, 10 h and then cells were collected and equal aliquots of fixed chromatin were subjected to ChIP precipitation with normal IgG, Brg-1, BAF155 and PML-II antibodies or Flag antibody, respectively. Precipitated chromatin DNA was assayed by SYBR green qPCR for testing (A) Brg-1 binding (B and C) BAF155, (D) PML-II and (E) PML-II- Δ RBCC binding at ISG54 or/and ISG56 promoter sequences. Results are presented as mean \pm SD of technical triplicate. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

4.2.11 Knockdown of PML-II decreases Brg-1 and BAF155 binding at gene promoter

Next, whether knockdown of PML-II affects Brg-1 and BAF155 binding at gene promoters was tested. As shown in Figure 4.2.11, the depletion of PML-II decreased Brg-1 and BAF155 binding at ISG54 and IFITM3 promoters (Figure 4.2.11 A). However, the removal of PML-II did not significantly reduce Brg-1 recruitment at ISG56 promoter, and also had no effect on BAF155 binding at either ISG56 or GBP1 (Figure 4.2.11B). Why did PML-II fail to affect BAF155 binding at these two promoters? Possibly this is because of individual ISG transcription/expression characteristics.

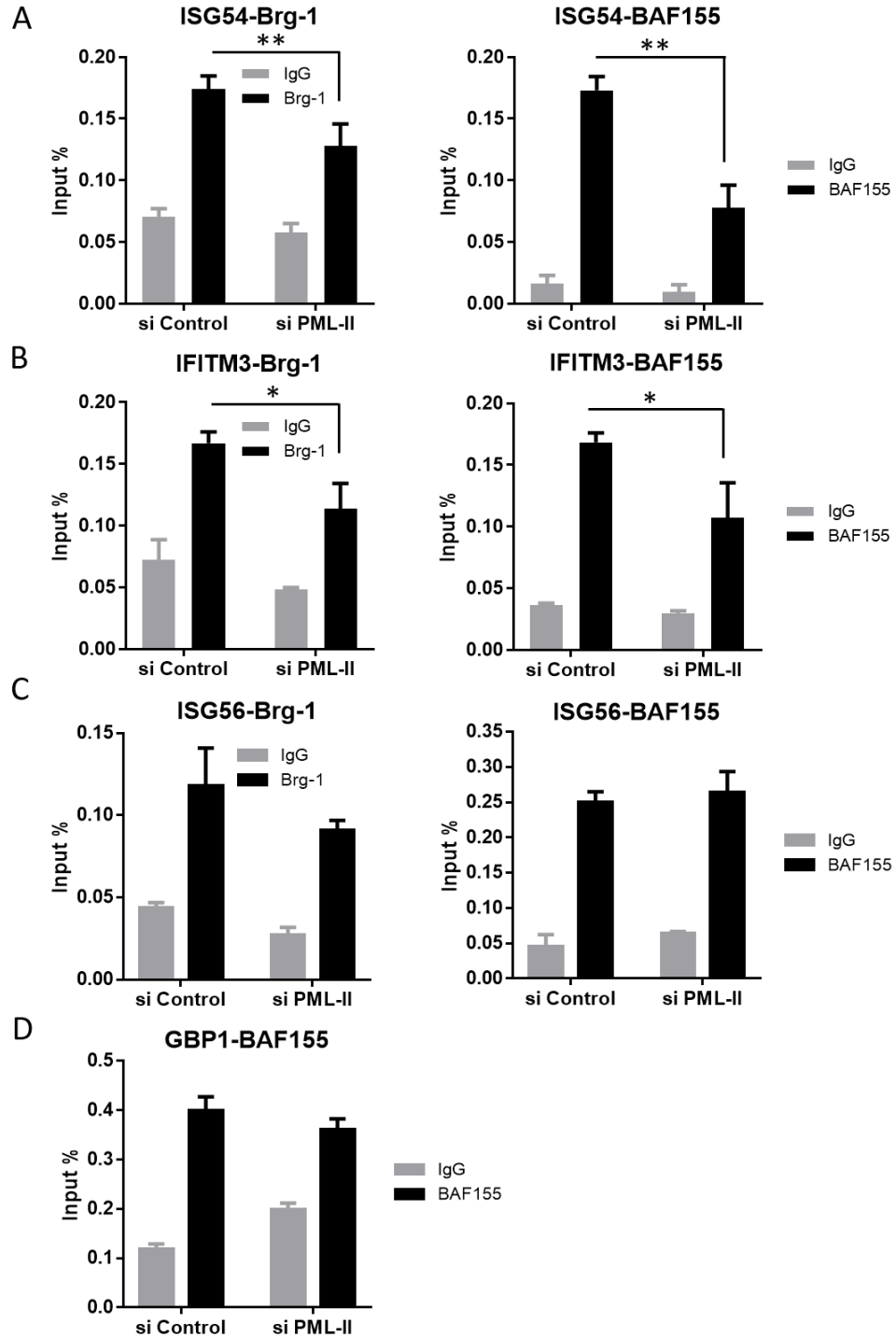


Figure 4.2.11 Knockdown of PML-II decreased Brg-1 and BAF155 binding at ISG promoters. Hela cells were transfected with 125 pmol/ml control, PML-II or Brg-1 si RNA for 48 h, then treated with 1000 unit/ml $\text{INF}\alpha$ for 6 h (Brg-1) or 8 h (BAF155), and then cells were collected and equal aliquots of fixed chromatin were subjected to ChIP precipitation with normal IgG, Brg-1 and BAF15 antibody respectively. Precipitated chromatin DNA was assayed by SYBR green qPCR for (A) ISG54, (B) IFITM3, (C) ISG56 and (D) GBP1 promoter sequences as indicated. Results are presented as mean \pm SD of technical triplicate. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

4.2.12 Different gene transcription/expression characteristics determines Brg-1 and BAF155 different binding pattern

ISGs generally can be divided into two groups, namely, the immediate & early responsive genes, and the later responsive genes (Ramirez-Carrozzi et al, 2009; Ramirez-Carrozzi et al, 2006). To test whether the differential effect of PML-II depletion correlated with ISG class, the transcription/expression pattern was tested on various ISGs including ISG54 and ISG56, together with two additional ISGs: ISG15 and GBP1 (Figure 4.2.12). Expression of ISG54 was activated and reached the highest expression level 2 hours quicker than that of ISG15, ISG56 and GBP1. This result confirmed that ISG54 belongs to the immediate & early responsive ISG class, while ISG15, ISG56 and GBP1 are from the later responsive class. This suggested that PML-II may have different influence on BAF155 recruitment patterns at promoters of these genes. As expected, the loss of PML-II indeed affected BAF155 binding at IFITM3 and ISG54 promoter and has no effect on GBP1 (Figure 4.2.11).

Accordingly, it can be concluded that PML-II regulates SWI/SNF complex chromatin remodeling function by decreasing subunit protein expression (BRM) and DNA binding/recruitment (Brg-1 and BAF155) at specific ISG promoters.

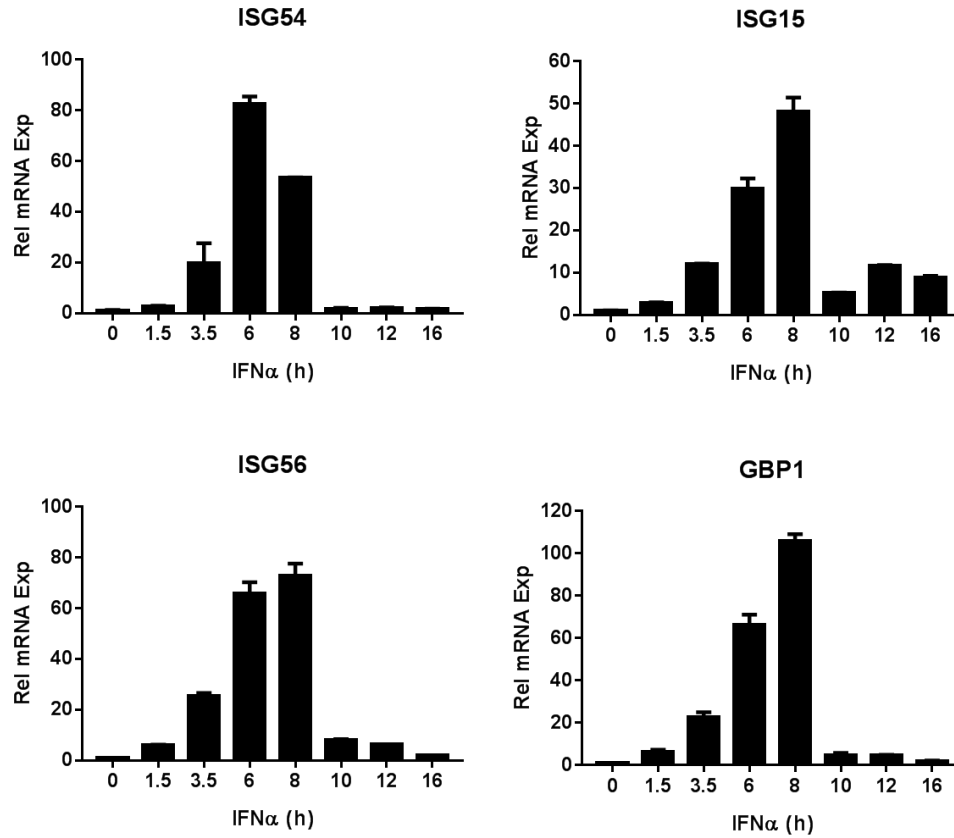


Figure 4.2.12 Different gene transcription/expression characteristics determines Brg-1 and BAF155 different binding pattern. Confluent Hela cells were stimulated with IFN α for 0h, 1.5h, 3.5h, 6h, 8h, 10h, 12 and 16h, then total RNA was extracted and subject to SYBR-Green RT-qPCR testing for various gene. The data shown are relative to no-stimulation and are the mean \pm standard deviation of three technique replicates

4.3 Discussion

4.3.1 ISG gene transcription and regulation by PML-II and Brg-1

In this chapter, the regulation function of PML-II on SWI/SNF Brg-1-containing transcription complex and histone post-translation methylation modification was studied. Consistent with our and others' previous reports, knockdown of either PML-II or Brg-1 decreased the expression of a large number of ISGs in response to IFN, including ISG54, ISG56, IFI27, IFI16 and GBP1 (Figure 4.2.2). Importantly, we also found that double-knockdown of PML-II and Brg-1 slightly enhanced the inhibition effect on the expression of these genes (Figure 4.2.6). This suggested a collaboration between PML-II and Brg-1 in gene transcription regulation.

Based on the gene expression timecourse testing, it was found that the mRNA expression of ISG54 reached the highest level at 6 hours after IFN α stimulation, then decreased gradually. This is consistent with characterization of primary-responsive ISG genes (also named immediate-early genes). In contrast, the mRNA level of genes such as ISG15, ISG56 and GBP1 reached the highest level at 8 hours which is 2 hours later than that of ISG54 6 hours. This may suggest that these genes belong to the later gene class (also named secondary-responsive gene). This result is quite similar to previous observation that some genes responded quickly while others responded slowly (Ramirez-Carrozzi et al, 2006). The different expression profiles of these genes may suggest a different regulation pattern in transcription. This study may imply different regulation model for Brg-1 and BRM in early and later ISGs, which may be helpful for understanding why different patterns of Brg-1 binding and regulation were observed in different genes.

The SWI/SNF remodeling complex in humans mainly includes the Brg-1-containing complex and the BRM-containing complex. Brg-1 has about 76% identity in amino acids with BRM indicating that functional activity of these two complexes may be similar to some extent. Both Brg-1 and BRM are implicated in the regulation of ISGs expression, and previous studies have indicated that the

requirement for Brg-1 or BRM in ISGs expression is different. For example, a IFITM3 gene reporter construct can be activated by both Brg-1 and BRM, this suggested this gene needs the overlap function of Brg-1 and BRM (Liu et al, 2002). In contrast, BRM is required by ISG56, IFITM3 and OAS1 for maintaining both basal and IFN α -induced expression (Lavigne et al, 2009), while Brg-1 is only partially required for ISG56 induction and unnecessary to maintain its basal expression. ISG15 expression is also less Brg-1 dependent (Liu et al, 2002).

In this study, the expression of ISG15, ISG56 and GBP1 was found reaching the highest level is about 2 hours later than that of the immediate-early genes such as ISG54 and IFITM3. This suggest Brg-1 and BRM may be responsible different in immediate-early and later ISG gene expression. Brg-1 is largely responsible for early/immediate ISG expression, while the later expressed genes are more likely regulated by BRM. In fact, biochemical assays have shown that the ATPase activity of Brg-1 is stronger than BRM (Kadam & Emerson, 2003). The difference of ATPase activity between Brg-1 and BRM might explain why early response gene induction, which is more likely regulated by Brg-1, is faster than slow response genes.

Brg-1 and BRM differentially regulate immediate-early and later-responding gene transcription, this may suggest a sequential recruitment and synergy function between Brg-1 and BRM protein. It therefore can be proposed that Brg-1 complexes quickly initiate early gene expression and leave the promoter, then BRM complexes are recruited after Brg-1 dissociate from the promoter and continue the remodeling process in the following time courses. Thus genes such as ISG54 and IFITM3 are more dependent on both Brg-1 and BRM, and their transcription can be started quicker by Brg-1 and maintained longer by BRM. In contrast the transcription of later genes like ISG15, ISG56 and GBP1 reaches the highest level later because they are less dependent on Brg-1 but more dependent on BRM. This may provide a mechanistic explanation for some cells retaining the overlapping function of Brg-1 and BRM-containing chromatin remodeling complexes, while others do not require Brg-1 (Kadam & Emerson, 2003; Liu et al, 2002; Ryme et al, 2009).

4.3.2 Association of PML-II with Brg-1 and TFs

SWI/SNF exists as a multiple protein complex. In this study, PML-II was found to associate with Brg-1 of SWI/SNF complex and STAT1 in response to IFN α stimulation. Brg-1 was also found to bind STAT1 suggesting PML-II might form a complex with SWI/SNF complex and STAT1. However, it is not sure whether the association of PML-II with Brg-1 is a direct event or indirect interaction as the co-IP technique is a technique that uses target protein-specific antibodies to indirectly capture proteins that are bound to a specific target protein. It would be of interest to conduct a further test by using GST-pulldown technique, which tests specifically for direct interactions, to clarify this outstanding question in the future.

In this study, the full-length PML-V isoform was also found to associate with Brg-1 under stimulation. This was shown to be probably through an indirect binding with other PML isoforms via their common RBCC structure because the RBCC domain-deleted form of PML-V lost the ability to bind Brg-1 and CBP, while the equivalent RBCC-deleted form of PML-II retained binding Brg-1 and CBP. This is consistent with previous observations, and also suggested an important function of the C-terminal fragment of PML-II in the association with Brg-1. Subsequently, a further analysis was conducted on PML-II C-terminal functional fragments. PML-II C-terminal 645-695 sequence fragment, especially 661-664 residue was necessary for this binding with Brg-1. Similarly, residue 645-695 was also very important for PML-II binding with CBP. The specific association of PML-II with Brg-1 and CBP suggested PML-II could have a role in chromatin remodeling/modification.

To further study the effect of PML-II on SWI/SNF complex stability/integrity, some co-IP experiments was performed to test whether the knockdown of PML-II impaired the interaction of Brg-1 with subunit BAF155 or transcription factor STAT1. However, the experimental background was always very high, so that it was not possible to make a conclusion. The reasons for this may be the interaction among SWI/SNF subunits is very tight, making SWI/SNF a very large molecular size pre-existing complex, so it is hard to demonstrate impaired

association among these proteins during transient knock-down experiments. Together, although it could not be concluded the effect of depletion of PML-II on the SWI/SNF complex stability, the observation that PML-II physically associated with Brg-1, STAT1 and CBP, suggesting PML-II is certainly involved in Brg-1-related transcription. This interaction between PML-II and Brg-1 suggested their collaboration on gene transcription.

4.3.3 Timing, order and interdependence of protein recruitment and histone modification at ISGs

In this study, as expected, STAT1 was not bound to ISG promoters in inactivate genes, however its binding was increased greatly by induction of IFN α , this supported that STAT1 activation is required for ISG gene expression as has been shown previously (Darnell, 1997; Darnell et al, 1994). Importantly, the enrichment of STAT1 binding at these promoters was closely associated with the mRNA expression from these genes, which was 1-2 hour later to reach the highest level than that of STAT1 binding. Similar to STAT1 association, the level of H3K4me3 modification at ISG promoters was also greatly increased by IFN α induction. Conversely, the level of H3K9me3 at promoters was decreased by IFN α . Interestingly, CBP binding was also increased which is very similar as that of H3K4me3.

It is notable that the temporal course of H3K4me3 modification was different in different genes. In ISG54 (early gene), H3K4me3 increased slowly in the first 6h after IFN α treatment and reached the highest level at 8 hours and decreased after then, while H3K4me3 in the ISG56 promoter underwent a trend of decrease during that period then rebounded to the highest level at 8h then quickly decreased. Loss of H3K9me3 was observed in ISG54 and ISG56 promoters upon IFN α stimulation that continued for the initial 6 hours after which amounts returned to those in unstimulated cells/basal level. The promoter specific differences of H3K4me3 disposition most likely is correlated with the differences, already discussed, in the way these genes interact with and are regulated by different forms of SWI/SNF complex containing the enzymatic core protein Brg-1 or BRM.

Similar to STAT1, CBP and H3K4me3 association with the promoters, the binding of PML-II, BAF155 and Brg-1 at ISG promoters was also increased by IFN α stimulation, and the maximum level of PML-II and BAF155 present at these promoters was at 8 h after stimulation, while the highest enrichment level of Brg-1 at the promoters was variable and dependent on the gene identity: for example, in ISG54 (early gene), Brg-1 binding reached its highest level at 6 hour after stimulation.

Taken together, the time post-stimulation that all these factors are recruited to promoter at the highest level is 2 hours late than the time of STAT1 DNA binding reaching its peak (4h-6h) and the induction of the highest amount of mRNA expression (6h-8h). This indicates that the maximum STAT1 binding is two hours earlier than that of the whole transcriptome assembly. The time of highest gene transcription level is consistent with the time of the whole transcriptional complex assembly, at 6h (early gene) or 8h (later gene) after stimulation. This suggested that transcription factor STAT1 activation and DNA binding is the most important determinant for transcription initiation of the genes tested here. However, the highest level of gene transcription requires full transcriptional complex assembly.

4.3.4 Sources of variation in ChIP signal strength for histone marks and proteins bound at ISG promoters

In this study, the occupation level of STAT1 and H3K4me3 at the promoter was very high while, in contrast, the binding level of Brg-1 was very low; similar phenomena were observed in CBP, BAF155 and PML-II binding. The variation of % precipitation at target gene promoters by different antibodies is unlikely to be due to variation in the expression of those proteins as the assay effectively reports on events at a chosen promoter across a large number of cells, within which there is unlikely to be large fluctuations in the expression of any given protein. Instead, this might reflect real differences in the proportions of target promoters carrying these modifications or bound proteins, or it might reflect technical differences such as antibody affinity. We have addressed several

possibilities that may contribute to this lower binding signal. For example, to discount the possibility that the antibody is not effective for immunoprecipitation experiments and to reduce nonspecific pulldown of other proteins, different antibodies were tested and the cell lysates were also precleared by salmon sperm affiliated beads to reduce the nonspecific binding. The gentle manipulation was also tried to avoid any interactions of Brg-1 with chromatin being disrupted by harsh washing buffers. Despite these efforts, the background signal in Brg-1 precipitations was very high but Brg-1 binding signal was very low. Potentially, the target epitope on Brg-1 might be masked as a consequence of variation with local experimental environments or in some complexes. Also, the affinity of SWI/SNF with DNA may also affect the ChIP efficiency. Although Brg-1 protein has a bromo domain, this motif has nothing to do with binding to promoter directly. The binding of SWI/SNF to the promoter requires other subunits of the SWI/SNF or DNA-binding proteins such as STAT1. This suggests that the binding of Brg-1 to DNA is via its association with other proteins.

Brg-1 might be working at more distant sites and have long-range effects on gene transcription. It has been described that Brg-1 has a long-range effect at the CIITA locus (Pattenden et al, 2002). One experiment was also carried out and found Brg-1 can bind not only at promoters, but also at far-downstream regions from the promoter with the same weak level, but the effect on upstream regions was not checked. It is possible that Brg-1 binding ability is stronger at the upstream region far from promoter.

In agreement with our observation, Ni (Ni et al, 2005) conducted an experiment testing the ChIP efficiency, and showed that histone has the most efficient DNA binding, followed by DNA-bound factors such as transcription factor and Pol II, the lowest binding efficacy being for cofactors that are recruited via protein-protein interaction. This is completely consistent with my results that the histone H3K4me3 has the highest binding efficiency (input %), next was STAT1, with the lowest binding proteins including PML-II, CBP, Brg-1 and BAF155 that associate with chromatin by protein: protein interactions.

4.3.5 Possible binding and regulation patterns of Brg-1 and PML-II

Various SWI/SNF complex (Brg-1) DNA binding models have been proposed, including the constitutive binding of SWI/SNF complex on an inactive ISG promoter to make it in a loose configuration, which allows STAT1 rapidly to target and bind the promoter upon stimulation, and so to activate transcription. That is, without prior chromatin remodeling by SWI/SNF complex, STAT1 may not efficiently bind the ISG promoter to induce gene transcription. Thus SWI/SNF was thought to be bound to promoter in silent genes independent of STAT1 (Ni et al, 2005), and that IFN α stimulation was not required for the BAF complex to bind (Cui et al, 2004; Liu et al, 2002). In other cases, specific DNA binding factors may be relevant to SWI/SNF recruitment. It was reported that SWI/SNF components can be detected in a multiprotein complex that contains both histone methyltransferases and HATs (Nakamura et al, 2002). For example, SWI/SNF and CBP/p300 interact with some DNA-binding proteins such as c-MYC, and Pol II in untreated cells, each of these factors is present at CIITA promoter IV (pIV) at detectable levels. Thus, these DNA-binding proteins may help tether Brg-1 and HATs to the silent pIV (Ni et al, 2005),

Data in this study firmly support the idea that STAT1 complexes play a role in constitutive Brg-1 recruitment to promoter and that is required for gene fully transcription/expression. One possibility is that the low level of SWI/SNF complex can be detectable in some gene promoters, for the maintaining the basal expression level of some early-responding gene, however, the gene highly activation and product expression must need STAT1 participation. It have been observed in the present study that STAT1 activation (30min phosphorylation) and recruitment to the promoter (4-6h) occur significantly earlier than the recruitment of Brg-1 and BAF155, also some other transcription coactivators. From this it can be concluded that the recruitment of Brg-1 to the promoter regions of selected ISGs and the activation of Brg-1 activity require additional transcription factors. Further studies are required for providing insight into the molecular basis of promoter specificity in Brg-1-mediated gene expression.

4.3.6 Why does knockdown of PML-II and Brg-1 have less effect on H3K4me3 at ISG promoters?

H3K4me3 is often considered to be a positive mark for transcription. The enrichment of H3K4me3 at ISG promoters was greatly increased by IFN α stimulation and the dynamics of H3K4me3 modification were closely matched to those of the other transcription-related factors including BAF155, CBP and PML-II (8h highest level). Depletion of PML-II or Brg-1 affected the binding of STAT1 and CBP and increased the level of H3K9me3 modification. However, the amount of H3K4me3 at these promoters was less affected by depleting PML-II or Brg-1, whereas it might have been predicted to be reduced. This is probably because H3K4me3 is not an essential component of an actively transcribed gene (Howe et al, 2017) and PML-II and Brg-1 depletion only have significant effects on transcription factor recruitment and of other proteins whose recruitment is transcription factor dependent. This may also because other pre-existing histone modifications such as acetylation on H3K4 at this site affect H3K4me3 enrichment/deposition at a promoter (Howe et al, 2017). Collectively, this result further emphasized a key role of STAT1, acting as a bridge in PML-II and Brg-1 recruitment and exerting regulation function on some other transcription-related factors. This suggested additional studies are required to clarify this question in the future.

4.3.7 How PML-II and Brg-1 affect the complex through STAT1 recruitment

Our and others' previous studies have suggested the recruitment of PML-II and Brg-1 at a promoter was STAT1 dependent and both proteins exerted functions via STAT1. The highest level of Brg-1 and PML-II enrichment at the promoter was 6h and 8h, respectively, while STAT1 DNA binding started from 2 hours and reached the highest level at 4h or 6h (depending on specific gene characteristics), which is 2 h earlier than Brg-1 and PML-II binding. In this case, it can be asked how do the later-recruited PML-II and Brg-1 proteins affect the earlier-arrived STAT1 binding, since PML-II or Brg-1 reduction clearly reduces STAT1 promoter binding. In fact, it can be seen that the formation of the whole transcriptional complex including PML-II, Brg-1, CBP and BAF155 was

maximal at about 8 hours after stimulation. At this time point, the level of STAT1 at the promoter was still very high compared to the basal level, although the level had started to decrease from the peak. This means there is a period when both Brg-1 and PML-II coexist with STAT1 at the promoter and may regulate its binding there.

Although depletion of either Brg-1 or PML-II reduced STAT1 binding at promoters, both proteins may function differently in the process. Depletion of Brg-1 impaired chromatin structure remodeling which would certainly increase the difficulty of STAT1 binding and thus inhibit STAT1-activated transcription. However, PML-II may function as scaffold protein for transcription complex formation. Therefore the knockdown of PML-II may impair the stability and integrity of the whole complex. Moreover, the depletion of PML-II decreased the expression of core SWI/SNF subunits such as BRM and BAF47 and could thus result in secondary impairment of the whole transcriptional complex through reduced SWI/SNF activity. This could then eventually affect STAT1 DNA binding.

4.3.8 The effect of PML-II on SWI/SNF complex and Brg-1/BAF155 recruitment

In this study, it has been demonstrated PML-II regulate the binding of Brg-1 and BAF155 at ISG promoters, however, it is notable that the influence of PML-II on these genes is not equal. PML-II has a stronger effect on Brg-1 and BAF155 binding at promoters of early genes such as ISG54 and IFITM3, while it has relative less effect on later genes including ISG56 and GBP1 (Figure 4.9A). Consistently, the dual-knockdown of Brg-1 and PML-II also showed the difference between earlier gene ISG54, and also IFN β , and later genes such as ISG15 and ISG56. It can be seen that knockdown of PML-II further inhibits the expression of IFN β and ISG54 by depleting Brg-1, while this effect was not observed in the later genes ISG15 and ISG56 (Figure 4.5). Considering the transcription of early gene is more likely regulated by BRM and Brg-1 (most likely in combination), while the expression of later gene is less dependent on Brg-1 (probably by BRM alone), it is not surprising that there is a smaller effect

of knockdown of PML-II on Brg-1 and BAF155 binding at promoters of these later response genes including ISG56 and GBP1 (Figure 4.2.11).

Collectively, these results suggest that the expression of the less Brg-1-dependent later genes may be more likely dependent on BRM activity, and therefore BRM may be the main subunit regulating their transcription. On that basis, knockdown of PML-II should have a significant effect on BRM binding at these promoters and this should be tested in the future.

Beside the effect of PML-II on Brg-1 and BAF155 binding at promoter, we have also observed that the loss of PML-II affected BAF47 and BRM gene expression. This suggested an alternative way for PML-II to exert a regulation function on SWI/SNF complex, because BAF47 is another important core subunit of the SWI/SNF chromatin remodeling complex and BAF47 down-regulation influences Brg-1 and BRM stability (Cui et al, 2004). The decreased expression of BAF47 and BRM would affect complex stability and integrity thus the binding at a promoter. The decreased expression of BRM due to depleting of PML-II is likely to impair BRM binding and BRM-containing complex stability. Therefore, regulating BAF47 and BRM expression by PML-II may also partly contribute to the influence on SWI/SNF complex function. Together, I conclude that PML-II regulates SWI/SNF remodeling complex function by affecting core subunits expression and/or its promoter binding.

4.3.9 Summary

In this chapter further evidence have been provided on PML-II functions in gene transcription. PML-II can form a complex with various transcription-related proteins including chromatin remodeling complex subunit Brg-1, transcription factor STAT1 and coactivator CBP, and these interactions are mediated by the PML-II C-terminal fragment. Depletion of PML-II impaired the binding of Brg-1, BAF155, STAT1 and CBP to ISG promoters. The SWI/SNF complex chromatin remodeling function was further affected by PML-II through downregulated levels of subunits BAF47 and BRM. PML-II and Brg-1 also demonstrated regulation function on histone H3K9me3 enrichment/deposition but had no effect on H3K4me3 recruitment. This suggested that PML-II is required for forming and stabilizing the whole transcriptional complex and hence

the depletion of PML-II impaired transcriptional complex stability and hence the binding of component proteins at a promoter.

Chapter 5 PML-II affects ERK and AKT signal activation and IFN α -mediated cell apoptosis

5.1 Introduction

Type I IFNs elicit pleiotropic biological effects including anti-virus, modulating immune responses and anti-tumour effects. Type I IFNs have been used for the treatment of several types of haematological malignancies and solid tumours (Ferrantini et al, 2007; Rizza et al, 2010). The expression products of a group of ISGs are thought to be the primary effectors that mediate the IFN antitumor function. Many ISGs have been identified that are related to this biological response, for example, classical ISG15 (Zhou et al, 2017), ISG54 (Reich, 2013; Stawowczyk et al, 2011), OAS1 and XAF-1 (XIAP associated factor-1), CD95 (Fas/APO-1), PML and RIDs (regulators of IFN induced death) (Chawla-Sarkar et al, 2003). The TRAIL/Apo2L (tumor necrosis factor-related apoptosis-inducing ligand) was also demonstrated to be an important effector required for IFN α -mediated growth inhibition and apoptosis in myeloma and hepatocellular carcinoma (Crowder et al, 2005; Herzer et al, 2009). Moreover, the expression level of PUMA, a p53-dependent gene, was also increased in response to IFN α stimulation in human myeloma cells suggesting a possibility of PUMA related to IFN α -induced apoptosis (Gómez-Benito et al, 2007).

Besides the increase of pro-apoptotic protein expression by IFN α stimulation, the inhibition of pro-survival signaling pathways such as ERK and AKT is also regarded as an important mechanism for IFN α -mediated antitumor function. IFN α treatment inhibited ERK signaling in transformed T cells and monocytes (Romerio et al, 2000). IFN α also transiently diminished the phosphorylation of both ERK and MEK in hepatocellular carcinoma (HCC) cell lines suggesting an inhibition on this pathway (Inamura et al, 2005). Recently, IFN α inhibition on AKT signaling was also observed in human cervical cancer cells (Ethiraj et al, 2016). PML, a tumour suppressor protein, has also been demonstrated to be involved in cell proliferation and apoptosis. The expression level of PML was low in various cancer cells compared to normal cells, and the deficiency of PML likely leads to tumorigenesis (Gurrieri et al, 2004; Trotman et al, 2006). The loss of *Pml* gene in a mouse model markedly accelerates tumour onset, incidence and progression (Trotman et al, 2006). Cells from PML-deficient mice show severe apoptotic defects, including a strongly decreased sensitivity to death receptor-

mediated apoptosis (Wang et al, 1998). It has been suggested that PML prevents cancer by inactivating nuclear AKT activity (Trotman et al, 2006).

Several studies have shown that PML is required for the efficient induction of apoptosis induced by type I and II IFNs (Quignon et al, 1998; Wang et al, 1998). Moreover, growth inhibition effects of IFN α in myeloma cells correlated with the presence of PML (Crowder et al, 2005). IFN α -induced apoptosis in hepatocellular carcinoma also involves PML (Herzer et al, 2009). However, the exact mechanism remains to be completely understood. Recent work in our laboratory demonstrated that PML-II regulates the type I IFN response and ISGs expression (Chen et al, 2015). This suggested that PML-II may also be involved in IFN α -mediated apoptosis. The purpose of this chapter is to dissect the role of PML-II in pro-survival ERK and AKT signaling pathway activation and IFN α -mediated apoptosis.

5.2 Results

5.2.1 Knockdown of PML-II downregulates anti-apoptotic ISGs expression

To investigate the biological role of PML-II in IFN α -induced apoptosis, the expression of PML-II itself in response to IFN α stimulation was studied. The *Pml* gene is an ISG because its promoter contains ISRE and GAS element sequences that make it possible for activation by IFN stimulation (Lavau et al, 1995; Stadler et al, 1995). As expected, the expression of PML-II was greatly increased and reached the highest level at 4 hours after stimulation (Figure 5.2.1 A). The expression level of PML protein is closely related to the induction of cell death and PML is one of the important ISG proteins that mediates IFN apoptosis function (Chawla-Sarkar et al, 2003; Quignon et al, 1998; Wang et al, 1998). The elevated expression of PML-II after IFN treatment may suggest a direct contribution of PML-II in IFN α -induced cell apoptosis. Importantly, the mRNA level of PML-II was reduced to undetectable when cells were treated with PML-II specific siRNA, and this inhibitory effect was not overcome by IFN α stimulation (Figure 5.2.1 A). Next, ISG15, ISG54 and OAS1 mRNA was

detected, expression of which was reportedly related to cell apoptosis (Reich, 2013; Stawowczyk et al, 2011; Zhou et al, 2017). Similar to PML-II, the expression of these genes was greatly increased by IFN α stimulation, and the level of ISG15 and ISG54 was greatly decreased by depleting PML-II. The expression of OAS1 was not so severely decreased as ISG15 and ISG54 but was still significantly decreased due to the loss of PML-II (Figure 5.2.1 B, C and D). This suggested that PML-II participates in IFN α -induced apoptosis probably through positive regulation on other pro-apoptotic ISG proteins.

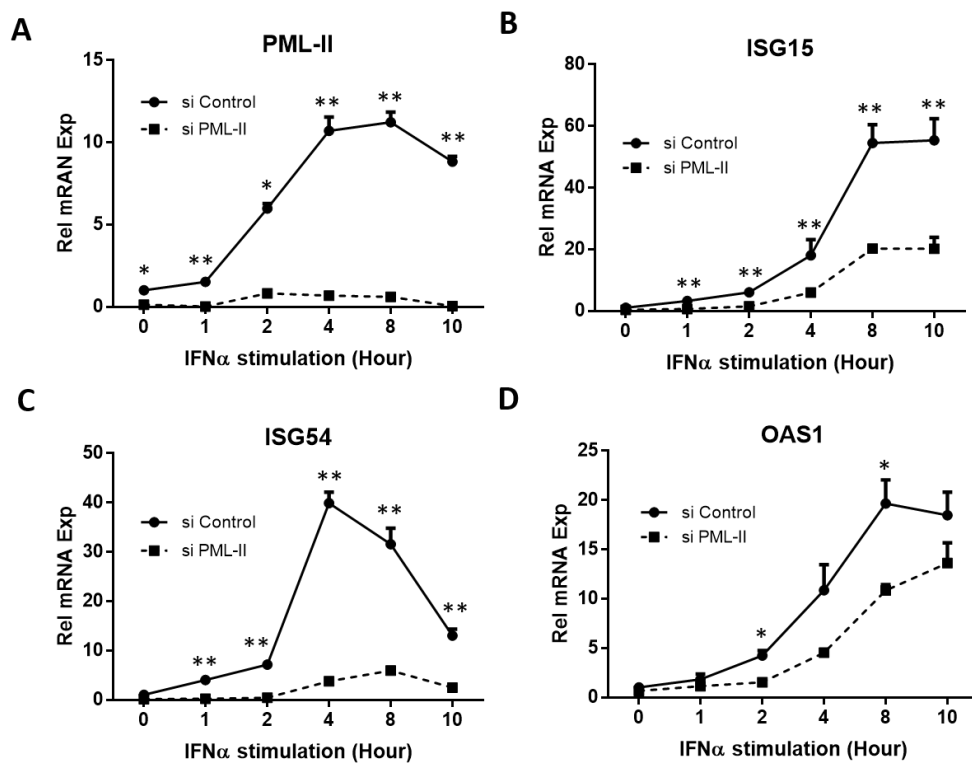


Figure 5.2.1 Knockdown of PML-II decreased pro-apoptotic protein expression. 1×10^5 Hela cells were plated at 24-well culture plate and grown to 30-50% confluence then transfected with 125 pmol/ml PML-II siRNA or control siRNA for 48 h. Cells were stimulated with 1000 U/ml IFN α and samples were collected at described time points. The mRNA levels of genes were measured by SYBR Green qPCR. The mRNA levels of (A) PML-II, (B) ISG15, (C) ISG54 and (D) OAS1 were quantified relative to GAPDH, based on Ct difference performed according to the “ $\Delta\Delta C_t$ ” method, and were then normalized to the level in control siRNA-treated, unstimulated cells. Results are presented as mean \pm SD of technical triplicate experiments. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

5.2.2 Knockdown of PML-II decreases IFN α -induced TRAIL expression

Besides the expression of classical ISGs, the direct induction of death receptor proteins such as TRAIL by IFN α and consequent activation of the extrinsic apoptosis pathway was also reported as one of the important means by which IFN induced cell apoptosis in melanoma and multiple myeloma (Crowder et al, 2005). In this study, the expression level of TRAIL was also observed quickly induced by IFN α stimulation at both mRNA level and protein level in Hela cells (Figure 5.2.2 A-C). The knockdown of PML-II greatly decreased the expression of TRAIL at both the mRNA and protein level (Figure 5.2.2 D-F). This result is consistent with a previous report that depletion of total PML downregulates TRAIL expression in hepatoma cells (Herzer et al, 2009) and suggests that PML-II is (one of) the most important isoforms that regulates IFN α -induced TRAIL expression. The regulation of IFN α -induced TRAIL expression suggests the involvement of PML-II in the death receptor-mediated apoptosis signaling pathway.

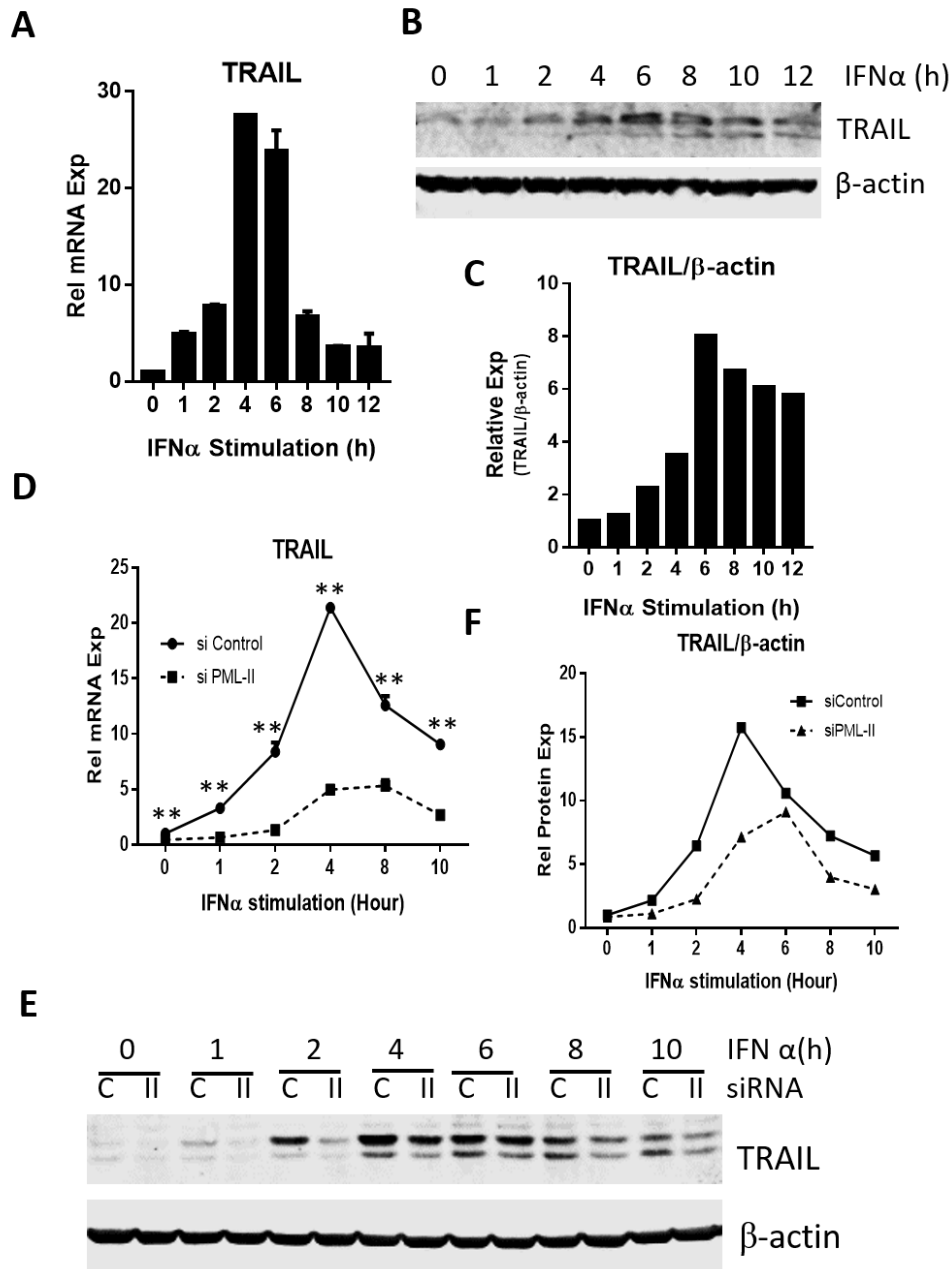


Figure 5.2.2 Knockdown of PML-II decreases IFNα-induced TRAIL expression. 1×10^5 HeLa cells were cultured in 24-well-plates and grown to 30-50% confluence then stimulated by 1000 U/ml IFNα and collected at different time points. Cell pellets were processed for total mRNA and protein, then analysed for TRAIL by qPCR (A) and WB (B), respectively. (C) The WB bands in panel B were visualized and quantified with an Odyssey system (Pierce). (D-E) HeLa cells were transfected with 125 pmol/ml PML-II siRNA or control siRNA for 48 hours then stimulated by 1000 U/ml IFNα and collected at different time points for testing mRNA (D) and protein (E) expression by SYBR Green qPCR or WB, respectively. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$. (F) The TRAIL protein bands in E were quantified with an Odyssey system (Pierce).

5.2.3 Knockdown of PML-II reduces IFN α -induced PUMA expression

In addition to the extrinsic apoptosis pathway, the mitochondrial-mediated intrinsic apoptosis pathway is also essential for cell apoptosis. PUMA is one of the important pro-apoptotic proteins of the mitochondrial pathway. Overexpression of PUMA resulted in an extremely rapid and profound apoptosis in colorectal cancer cells (Yu et al, 2001). IFN α treatment increased the level of PUMA in multiple myeloma (MM) (Gómez-Benito et al, 2007). Therefore, the effect of IFN α stimulation on PUMA expression was tested in HeLa cells. It was found that the transcripts of pro-apoptotic PUMA and BAK were induced following IFN α treatment although this induction was very much less than that of ISGs and TRAIL (Figure 5.2.3 A-C). However, IFN α stimulation had no effect on the expression of BCL-2 family pro-survival proteins such as Bcl2 and Bcl-xL (Figure 5.2.3 D and E). Similar to other ISGs, the depletion of PML-II significantly decreased the expression of PUMA at the mRNA level (Figure 5.2.3 B) and protein level (Figure 5.2.3 F and G).

Collectively, the observations that PML-II regulates both IFN α -induced PUMA and TRAIL expression suggested that PML-II is an important participant/regulator in both mitochondrial-mediated apoptosis (PUMA) and death receptor-mediated apoptosis (TRAIL).

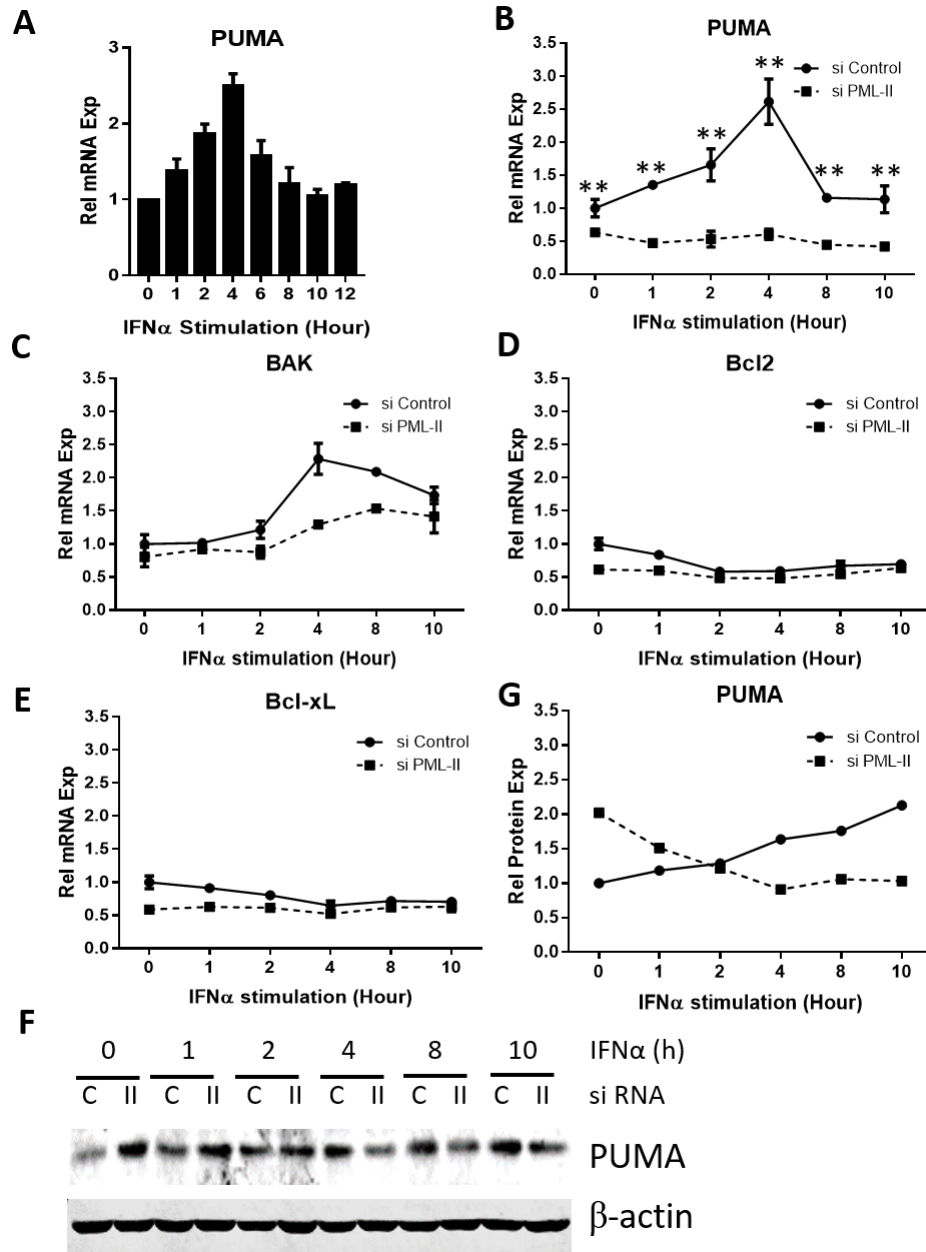


Figure 5.2.3 Knockdown of PML-II decreases IFN α -induced PUMA and BAK expression. (A) 1×10^5 HeLa cells were seeded in 24-well-plates and grown to 80% confluence then stimulated by 1000 U/ml IFN α at different time points, samples was collected, the relative expression of PUMA by SYBR-Green PCR. (B-F). HeLa cells were cultured as (A) and when grown to 30-50% confluence, cells were transfected with 125 pmol/ml PML-II siRNA or control siRNA for 48 hours then stimulated by 1000 U/ml IFN α . At different time points, the relative mRNA expression of (B) PUMA, (C) BAX, (D) BCL2 and (E) BCL-XL was tested by SYBR-Green qPCR. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$. (F) PUMA protein level was detected by WB. (G) The PUMA protein bands in F were quantified with an Odyssey system (Pierce).

5.2.4 PML-II regulates transcription factor p53 and STAT1 expression and activation

Next, the mechanism of how PML-II regulates the transcription of these pro-apoptotic genes was investigated. Transcription is strictly regulated by specific transcription factors. STAT1/2 is the most important transcription factor for ISGs expression by binding to the ISRE element in ISG promoters. PUMA is a well-known p53-dependent gene but it can also be induced in response to IFNs (Gómez-Benito et al, 2007). The TRAIL promoter sequence also contains various binding sites for the recruitment of TFs such as p53 and NF- κ B, but also has both ISRE and GAS element sequences (Wang et al, 2000). Therefore the effect of IFN α and PML-II depletion on p53 and STAT1 expression and activation was tested. The expression level of STAT1 was gradually increased by IFN α which is consistent with previous report that STAT1 is an ISG (Figure 5.2.4 A-C) (Lehtonen et al, 1997; Matikainen et al, 1999). The phosphorylation level of STAT1 was undetectable in unstimulated HeLa cells, however, upon stimulation by IFN α the level of p-STAT1 was quickly and greatly increased to the highest level at 30 minutes after stimulation (Figure 5.2.6A). It then gradually decreased in the following hours but still remained at a relatively higher level than the non-stimulated control (Figure 5.2.4 A, B, F and G). Interestingly, the total level of STAT1 was slightly decreased by depleting PML-II (Figure 5.2.4 A and B) and consequently the level of p-STAT1 was also decreased (Figure 5.2.4 F and G). This is probably because STAT1 itself is an ISG, and hence its expression is regulated by PML-II.

A previous report showed that in mouse embryonic fibroblasts (MEFs), IFN α/β stimulation could induce p53 expression but could not induce p53 activity (Takaoka et al, 2003). In HeLa cells, p53 expression level is low, because of the presence of the human papillomavirus E6 protein, and the phosphorylation level of p53 was undetectable in unstimulated HeLa cells (Figure 5.2.4 A and F). IFN α stimulation did not apparently increase p53 expression but greatly increased p-p53 level (Figure 5.2.4 F). This observation is different from the previous report of Takaoka (Takaoka et al, 2003), probably because the cell type and IFN utilized in the two studies are different. The earlier study used MEF cells and

IFN- β stimulation, while in my study, HeLa cancer cell was used and stimulated with IFN α .

Knockdown of PML-II decreased the expression of p53 at the mRNA and protein levels, while there was no obvious effect of depleting PML-II on p53 phosphorylation (Figure 5.2.4 E, F and H). In this case, how could the transcription and expression of PUMA be significantly affected by depleting PML-II? This may be because the phosphorylation of p53 is not essential for PUMA gene transcription but rather that other post-translational p53 modifications such as acetylation determine p53 activation and p53-dependent gene transcription. Altogether, these data suggest that regulation of transcription factor p53 and STAT1 expression and/or their post-translational modification may be one of the mechanisms by which PML-II regulates ISGs, TRAIL and PUMA gene expression.

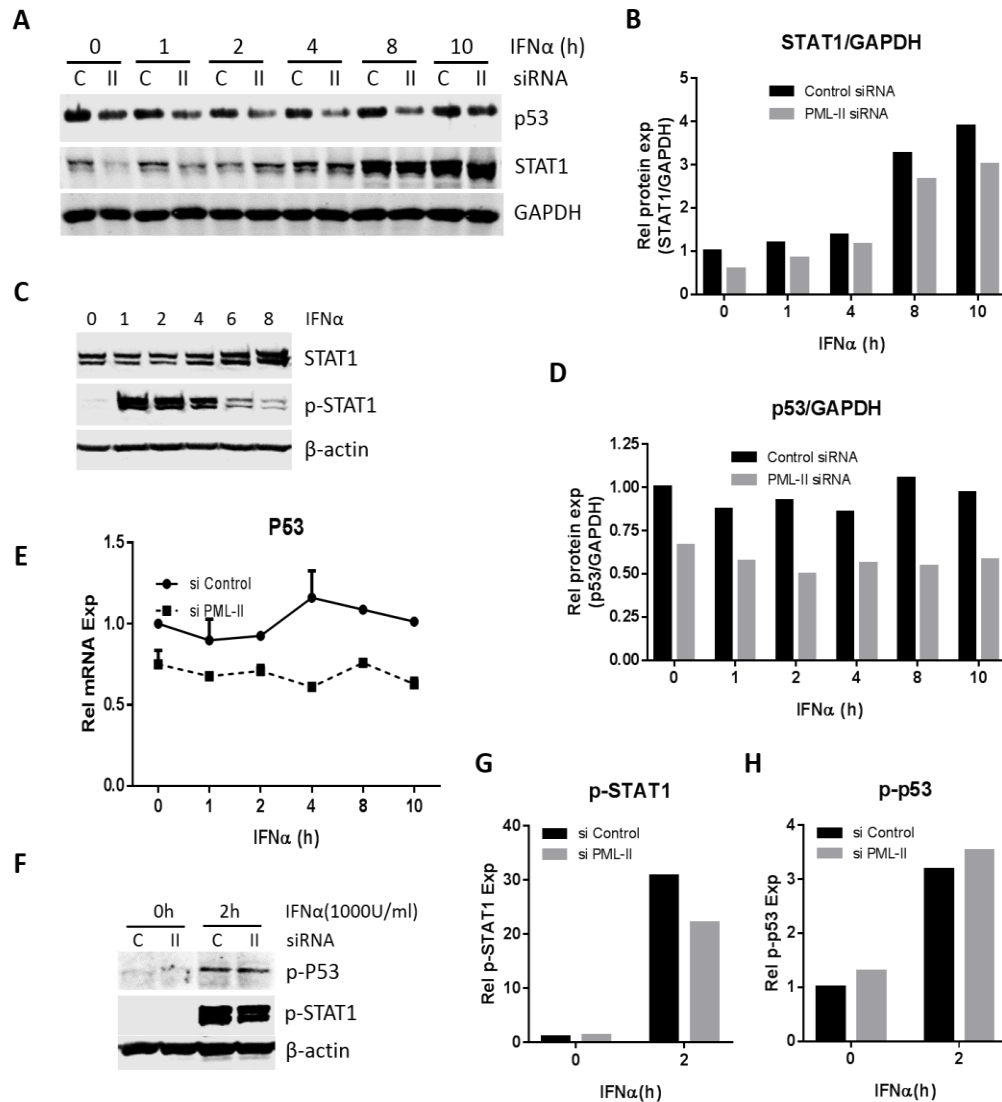


Figure 5.2.4 PML-II regulates transcription factor p53 and STAT1 expression and phosphorylation. (A) 1×10^5 HeLa cells were seeded into 24-well-plates and grown to 30-50% confluence, transfected and stimulated as Figure 5.2.3 F, then protein detected for p53 and STAT1 by WB. (B) Protein bands of total STAT1 in (A) were quantified with an Odyssey system (Pierce), normalized to internal control GAPDH, and the value was presented relative to control siRNA without stimulation. (C) HeLa cells were treated as Figure 5.2.2B, and the level of phosphorylated STAT1 was tested by WB. (D) Protein bands of total p53 in (A) were quantified as in (B). (E) The mRNA level of p53 in HeLa cells treated as in (A) was analyzed by SYBR Green qPCR. (F) 1×10^5 HeLa cells were cultured in 24-well-plates to 80% confluence and stimulated with 1000 U/ml IFN α then, at the time points shown, phospho-STAT1 and phospho-p53 were detected by WB. (G, H) Protein bands in (F) of phospho-STAT1 (G) and phospho-p53 (H), were quantified as in (B).

5.2.5 PML-II affects transcription factor binding at gene promoters

We recently demonstrated that PML-II can bind to various TFs and affect their recruitment at gene promoters (Chen et al, 2015). Therefore the effect of PML-II on p53 and STAT1 binding at these pro-apoptotic gene promoters was tested. As expected, IFN α stimulation increased p53 binding at PUMA, and STAT1 binding at ISG54 and OAS1 gene promoters. The knockdown of PML-II considerably decreased STAT1 binding at ISG54 and OAS1 gene promoters. This is consistent with our previous observation in ISG15 and ISG54 genes (Chen et al, 2015). Similarly, p53 binding at the PUMA promoter was also significantly affected by depletion of PML-II. Taken together, these results indicated that PML-II regulates expression of this pro-apoptotic protein probably through affecting TFs expression, activation or DNA binding. This provided a mechanistic explanation for PML-II involvement in IFN α -mediated apoptosis.

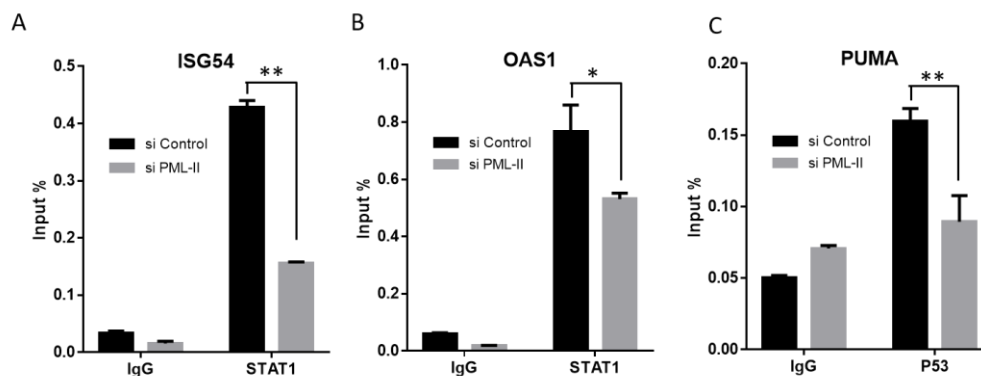


Figure 5.2.5 Knockdown of PML-II affects p53 and STAT1 binding at gene promoters. 1×10^6 HeLa cells were cultured in 10 cm dishes and grown to 30-50% confluence, then transfected with 125 pmol/ml PML-II or control siRNA for 48 h, and stimulated with 1000 U/ml IFN α for 8h, ISG54 (A) or OAS1 (B) and for 4 h, PUMA (C), then chromatin was subjected to ChIP with STAT1 and p53 antibodies. Precipitated DNA was assayed for ISG54, OAS1 and PUMA promoter sequence by SYBR Green qPCR. Results shown are the means \pm SD of technical triplicate experiments. One tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

5.2.6 IFN α inhibits AKT signaling but has no effect on ERK signaling in HeLa cells

ERK and AKT signaling pathways are essential for cancer cells acquiring proliferation and survival signaling. Inhibiting both pathways accordingly promotes cell apoptosis. As discussed in section 5.1, previous studies have shown that IFN α can reduce both ERK and AKT signaling, therefore the effect of IFN α on these signaling pathways in the context of PML-II depletion was tested. Initially, the effect of IFN α on these pathways was characterized in HeLa cells. As shown in Figure 5.2.6 A, upon stimulation of HeLa cells by IFN α the phosphorylation level of STAT1 was quickly increased, suggesting stimulation was successfully. However, IFN α stimulation did not transiently alter p-ERK level in the first hour (Figure 5.2.6 A). There was no apparent change in ERK phosphorylation in the following several hours, and even after 24 or 72 hours exposure to IFN α at the concentrations of 1000 U/ml and 5000 U/ml (Figure 5.2.6 B, C and E). This visual impression was confirmed by band quantitation relative to the loading control (Figure 5.2.6 D and F). Similar to p-ERK, the phosphorylation level of MEK, the upstream signaling component of the ERK pathway was also less affected by IFN α stimulation (Figure 5.2.6 C-F).

Similar to its lack of effect on total ERK, IFN α stimulation did not change the total level of AKT. However, IFN α treatment inhibited AKT phosphorylation in a dose-dependent pattern after 24 hours IFN α treatment. This inhibitory effect on AKT phosphorylation was also observed with a 72 hours IFN α stimulation (Figure 5.2.6 E and F). This is consistent with the recent observation that IFN α inhibited AKT signaling in human cervical cancer cells (Ethiraj et al, 2016). Altogether, these data demonstrated that IFN α inhibits AKT pro-survival signaling in HeLa cells and may suggest that inhibition of the AKT pathway by IFN α makes an important contribution to IFN α -induced apoptosis.

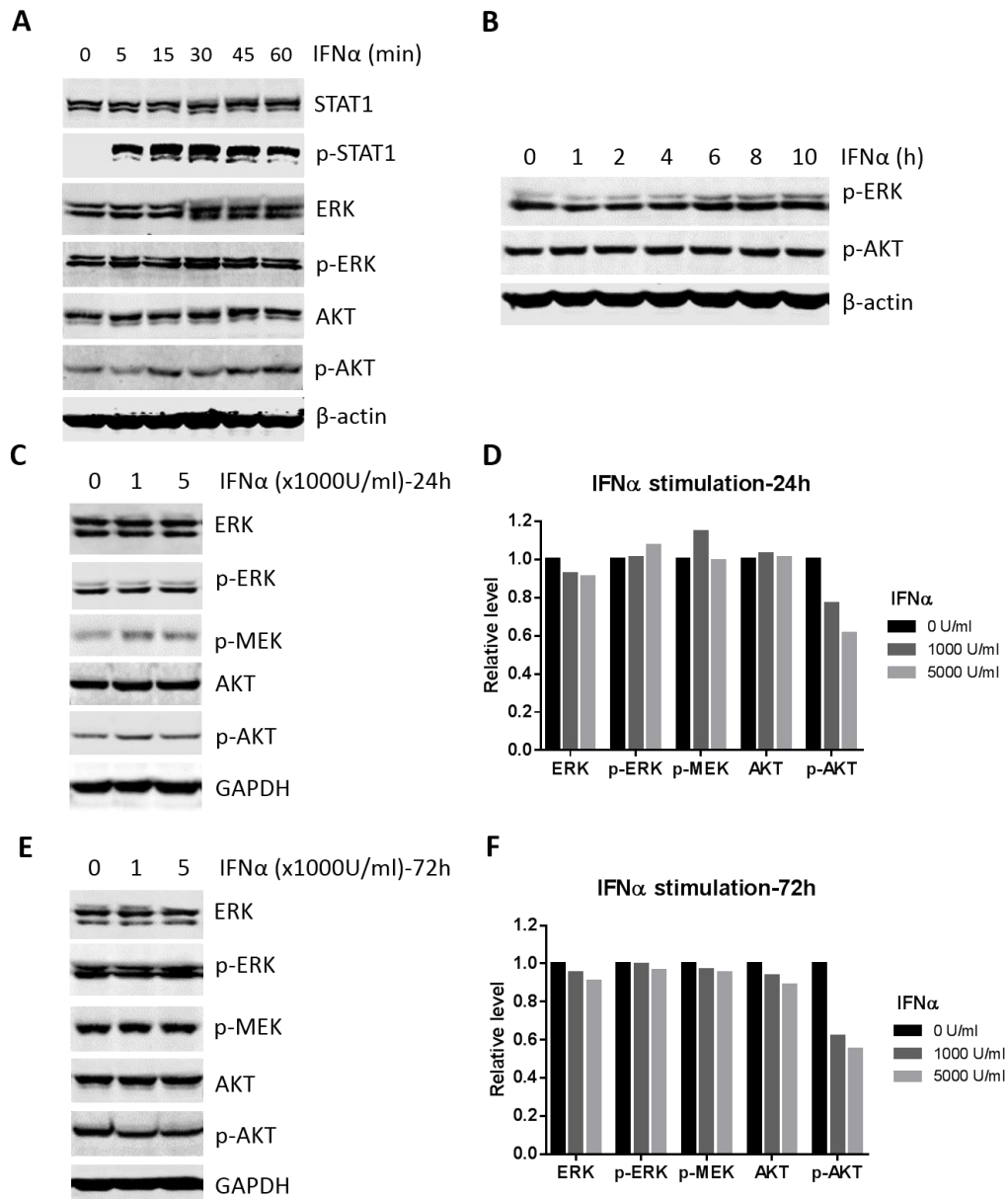


Figure 5.2.6 IFN α stimulation decreases AKT but not ERK signaling pathway activation. (A-B) 1×10^5 HeLa cells were cultured in 24-well-plates and grown to 80-100% confluence, then stimulated with IFN α at 1000 U/ml, cells were collected at the described time points and lysed for detecting appropriated proteins by WB. (C) HeLa cells were cultured to 30-50% confluence and stimulated with 1000 U/ml or 5000 U/ml IFN α or no stimulation, for 24 h, then cells were lysed for WB testing. (D)] he graph shows the quantification of WB bands in (C) by using the Odyssey system (Pierce), normalized to GAPDH and expressed relative to non-IFN α stimulation. (E) HeLa cells were cultured as (C) and stimulated with IFN α for 72 h. (F) The graph shows the quantification of WB bands in (E).

5.2.7 Knockdown of PML-II enhances ERK and AKT signaling

Given the biological role of PML in cancer cell proliferation and apoptosis (Crowder et al, 2005; Herzer et al, 2009; Wang et al, 1998), the effect of PML-II depletion on ERK and AKT signaling pathways was tested. The knockdown of PML-II increased ERK phosphorylation level, suggesting an enhancement of ERK signaling (Figure 5.2.7 A). To further confirm this, the expression of ERK-dependent genes c-Fos and c-Myc was tested. As expected, the depletion of PML-II indeed upregulated the expression of these ERK-dependent genes (Figure 5.2.7 B). The phosphorylation level of AKT was also increased by depleting PML-II (Figure 5.2.7 A); this is consistent with a previous report that PML prevents cancer by inactivating p-AKT inside the nucleus (Trotman et al, 2006). Moreover, the depletion of PML-II also increased the phosphorylation level of p70S6K1, a downstream effector of AKT, suggesting PML-II is an important isoform in the suppression of AKT signal activation in cancer cells (Figure 5.2.7 C). Collectively, these data suggested that PML isoform II negatively regulates ERK and AKT signals in HeLa cells, which provides further evidence of PML protein functions in tumor suppression.

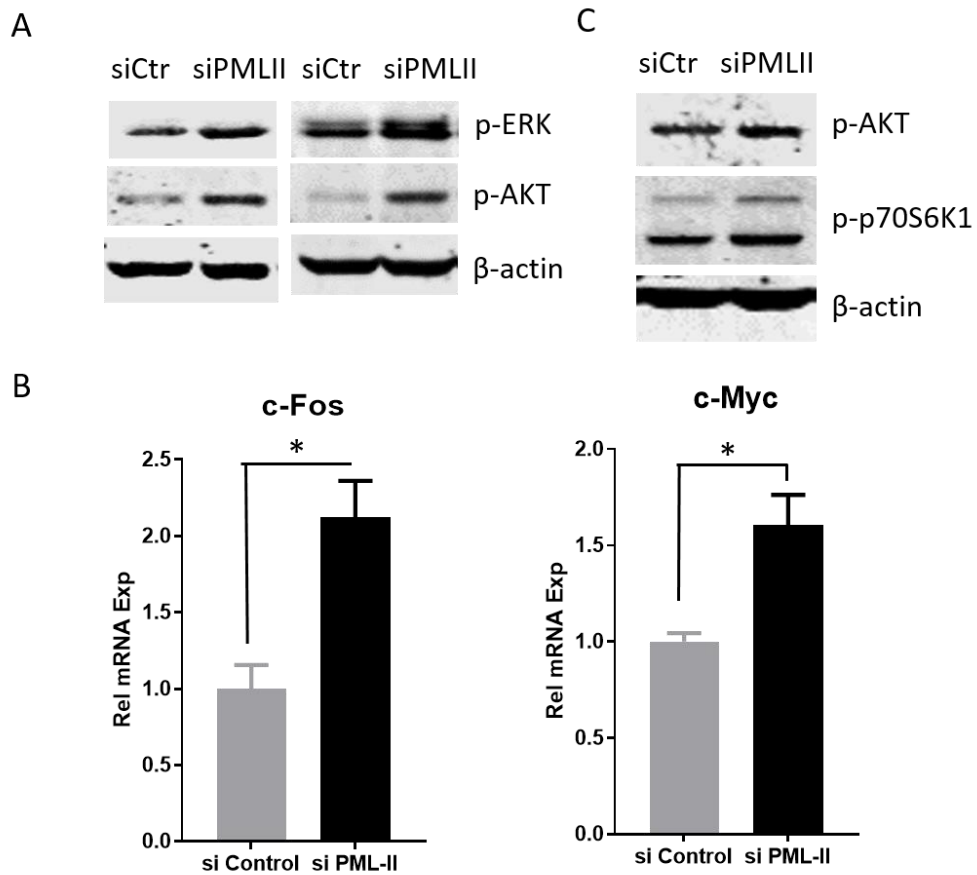


Figure 5.2.7 Knockdown of PML-II promotes ERK and AKT activation. (A) HeLa cells were transfected with 125 pmol/ml PML-II siRNA or Control siRNA, and after 48 h, cells were lysed and the expression of p-ERK and p-AKT proteins was detected by WB. β -actin was used as loading control. Two independent experiments were conducted and shown here. (B) Cells were treated as (A), and the mRNA expression of c-Fos and c-Myc genes tested by SYBR-Green qPCR. (C) HeLa cells were treated as (A), and phospho-p70S6K1 was detected by WB as (A). (D) Cells were transfected with siRNA as (A), then following a 1000 U/ml IFN α stimulation, cells were collected at various time points as described and cell pellets were lysed for detecting p-ERK and p-AKT protein by WB; β -actin was used as loading control. Two tailed student t-test; * $p < 0.05$, ** $p < 0.01$.

5.2.8 Knockdown of PML-II increases cell resistance to IFN α -mediated apoptosis

In this study, it has been demonstrated that PML-II positively regulates IFN α -induced anti-apoptotic protein expression and meanwhile negatively modulates pro-survival AKT and ERK signaling in HeLa cells. It was of interest therefore to assess the biological significance of PML-II in IFN α -induced cancer cell apoptosis.

Before testing cell death, the effect of PML-II on IFN α -mediated inhibition of AKT signaling was tested. Similar to Figure 5.2.6 C and D, IFN α stimulation decreased AKT phosphorylation in a dose-dependent pattern (Figure 5.2.8 A and B). As expected, the depletion of PML-II partly recovered/increased the level of p-AKT and this was less dependent on IFN α stimulation (Figure 5.2.8 A and B). To conduct apoptosis experiments, the efficiency of IFN α induced apoptosis was firstly tested in HeLa cells. Dead cells were stained with both Annexin-V and propidium iodide (PI) for testing the different types of cell death. The number of cells positive for Annexin-V and PI staining was increased in an IFN α -dose dependent pattern. This suggested cell death was due to IFN α -mediated apoptosis. Notably, the total death rate of HeLa cells exposed to 1000 U/ml IFN α for 24 hours was very low, although higher concentration IFN α (5000 U/ml) stimulation did induce some HeLa cell death (Figure 5.2.8 C). Next, the effect of PML-II on IFN α -induced cell apoptosis was tested. As expected, the knockdown of PML-II increased cell resistance to IFN α -induced apoptosis at every concentration tested although with a modest extent (Figure 5.2.8 D).

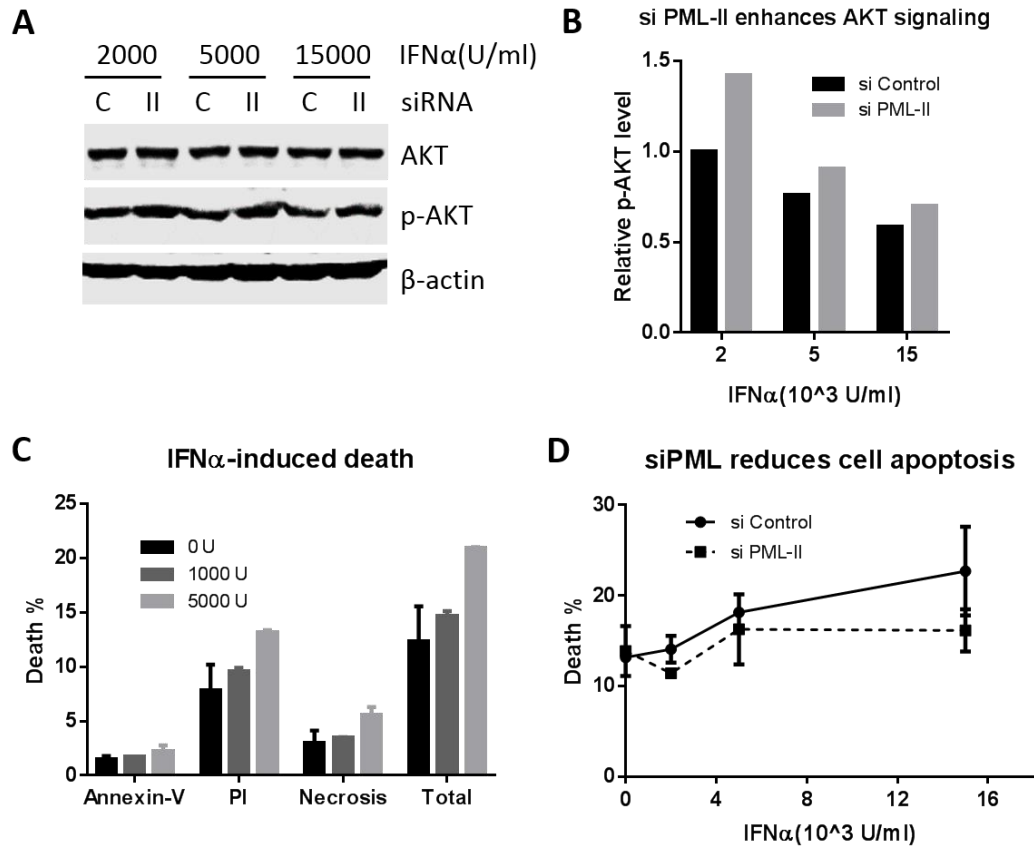


Figure 5.2.8 Effect of IFN α stimulation on cell apoptosis and p-ERK and p-AKT signaling. (A) HeLa cells were cultured and transfected with either siPML-II or control siRNA for 48 h, and following IFN α stimulation at 2000 U/ml, 5000 U/ml or 15000 U/ml for 24 h, cells were collected for WB detecting p-AKT. (B) The graph shows the quantification of protein bands in (A). (C) HeLa cells were cultured as Figure 5.2.6 A, and stimulated with 1000 U/ml or 5000 U/ml IFN α for 24 h, then cells were collected and stained with Annexin V and PI for testing cell early apoptosis and later stage death. (D) HeLa cells were treated with siRNA as (A), treated with 1000 U/ml for 24 h, and cells were collected and stained by PI for FACS analysis to determine the proportion of cell death in the population.

5.3 Discussion

5.3.1 PML-II regulate IFN α -mediated apoptotic protein expression

In this Chapter, we have confirmed that IFN α stimulation increases the expression of many pro-apoptotic proteins including ISG15 (Zhou et al, 2017),

ISG54 (Reich, 2013; Stawowczyk et al, 2011), OAS1 (Chawla-Sarkar et al, 2003). The expression of PUMA and TRAIL was also greatly increased at both mRNA and protein levels by IFN α in HeLa cells, which suggested an activation of both intrinsic and extrinsic caspase-mediated cell apoptosis pathways. This is consistent with the previous observation that the induction of TRAIL by IFNs increases cell sensitivity to IFN treatment in melanoma cell lines (Chawla-Sarkar et al, 2001).

It is notable that the expression of some pro-apoptotic genes such as ISG54 (Figure 5.2.1A), PUMA (Figure 5.2.3A) and TRAIL (Figure 5.2.2A) quickly reached the highest level at 4 hours after stimulation, while the level of ISG15, PML-II and OAS1 reached the highest level later, at 8 hours after exposure to IFN α (Figure 5.2.1A). This may be because some genes play roles in the immediate/first phase of an IFN response, while others are secondary response genes responsible for the later stages. It was also noted that the expression of ISG54 and PUMA decreased again very quickly, while other proteins including TRAIL remained at a relative high level for a longer time. The mechanism by which some proteins are turned over (degraded) more rapidly than others in the context of IFN α treatment remains unclear. Together, the greatly increased expression of a group of pro-apoptotic ISGs, PML, PUMA and TRAIL by IFN α should contribute to IFN α -mediated apoptosis/death or inhibition of cell proliferation.

In this study, PML-II was also demonstrated is required for the efficient IFN α -induced expression of these pro-apoptotic genes, as knockdown of PML-II greatly decreased the expression of these genes. The downregulation of TRAIL by depleting PML-II is consistent with a previous observation that loss of PML decreased TRAIL expression in hepatocellular carcinoma cells (Chawla-Sarkar et al, 2001). The inhibition effect of IFN α on myeloma cell growth correlated with PML-NBs and TRAIL induction (Crowder et al, 2005). RNAi silencing of PML downregulated TRAIL expression in hepatoma cells and correspondingly blocked IFN α -induced apoptosis (Herzer et al, 2009). All these results suggest that TRAIL is essential in IFN α -induced growth inhibition or apoptosis and also

suggest that PML has an important regulation function in IFN α -induced TRAIL expression.

Besides its effects on TRAIL, PML-II regulates IFN α -induced PUMA expression was also demonstrated for the first time in this study. PUMA is an important effector in mitochondria-mediated cell apoptosis. Downregulating PUMA and TRAIL expression by depleting PML-II may be essential for mediating caspase activation and thus inducing apoptosis. It was notable that in the unstimulated situation, PML-II siRNA treated cells had a relatively higher level of PUMA than control treated cells. This is similar to the effect of PML knockdown on TRAIL in myeloma cells (Crowder et al, 2005) though the mechanism is unknown. Together, silencing PML-II greatly downregulated the expression of PML-II itself and many other ISG proteins. Decreased PUMA and TRAIL expression provides a mechanistic explanation for why PML is required for IFN α -induced apoptosis/anti-tumor activity, though the documented involvement of PML-IV in p53 activation is also highly relevant (Fogal et al, 2000; Guo et al, 2000a).

5.3.2 The effect of IFN α and PML-II on STAT1 and P53, and recruitment at promoters

Previous studies have shown that the p53 gene can be transcriptionally induced by IFN β in mouse embryonic fibroblasts (MEFs): IFN β stimulation activates ISGF-3 and promotes its recruitment to the ISRE element sequence in the p53 gene promoter, leading to an increase in p53 mRNA and protein levels. Importantly, IFN α/β treatment itself does not directly activate p53, but contributes to boosting p53 responses to other stress signals (Takaoka et al, 2003). In the present study, a significant increase in p53 expression by IFN α stimulation was not observed in HeLa cells. However, IFN α stimulation greatly increased p53 phosphorylation level although the basal level of phosphorylated p53 was extreme low to undetectable in unstimulated HeLa cells. The relatively low level of p53 and p-p53 in HeLa cells is because papillomavirus HPV18 E6 protein causes p53 degradation in HeLa cells so p53 protein remains at a low level even under conditions of IFN α stimulation. The difference in expression

and activation between both studies is probably because the cell types were different and IFNs utilized were different, and thus different PAMPs recognition receptors (PRR) were involved. In contrast to p53, the total level of STAT1 was gradually increased and the phosphorylation level of STAT1 was immediately increased after IFN α stimulation to the highest level at about 30 minutes, and then followed a gradual decrease in the following several hours although the level of p-STAT1 was increased due to the total STAT1 level increasing upon prolonged IFN α stimulation.

The depletion of PML-II decreased the expression of total p53 and STAT1 at the protein level and the mRNA level, and the phosphorylation level of STAT1 was decreased while the level of p-p53 was not significantly affected. Each phosphorylation is carried out by a specific kinase that is activated in response to a particular stimulus. P-p53 then favors further acetylation modification because phosphorylation prevents p53 interaction with MDM2 and hence stabilizes the protein (ie prevents its MDM2-dependent degradation). Therefore an IFN-induced increase in expression is effectively equivalent to phosphorylation in providing a greater pool of p53 protein that can then form tetramers and be acetylated (the DNA-binding transcription activator form of the protein). IFN α stimulation affects the acetylation level of p53 but the effect on the phosphorylation level is less clear, actually it is the acetylation level of p53 that is essential of p53-dependent gene transcription.

The mechanism of PML-II regulating gene expression was further investigated, following the previous observation in our laboratory that knockdown of PML-II affects STAT1 binding at ISG54 and OAS1 gene promoters. The binding of p53 at the PUMA gene promoter was also decreased by depletion of PML-II. This is different from our previous work (Chen et al, 2015) which showed that PML-II knockdown had no effect on (or slightly increased) p53 binding to the p21 promoter in response to etoposide (DNA damage) stimulation in HEK293 cells, which was consistent with the slight increase in p-p53 due to depleting PML-II observed in this study. The reason why PML-II has different effects on p53 binding at these two p53-dependent genes could be because the genes are distinct p53-responsive gene classes (p21, cell cycle arrest class; PUMA, apoptosis

class). It may also be because the cell types and mode of stimulation are different.

The decreased total STAT1 and p53 expression caused by depleting PML-II may also partly have impaired TF binding at promoters and thus possibly activation, however, we propose that the direct effect on TF binding by knockdown of PML-II contributes the most effect on gene expression. In summary, PML-II can regulate gene expression through multiple direct or indirect means, including decreased total TF level expression, impaired TF activation and impaired DNA binding.

5.3.3 IFN α stimulation has no effect on ERK signaling in HeLa cells

Many previous studies have demonstrated the link between IFNs and ERK signaling. Some reports showed that IFN α stimulation transiently activated ERK signaling (Arora et al, 1999; David et al, 1995; Stancato et al, 1997), and the regulation of MAPK activity is also essential for cytokine-induced expression of immediate-early ISG. The reason for the transient activation of ERK signaling by IFN α probably is because of the crosstalk between the JAK-STATs pathway and the RAF/MEK/ERK pathway. However, some contradictory results have also been reported in other studies: IFN α treatment reduces the phosphorylation of MEK1 and ERK1/2 in transformed human hepatocellular carcinoma (HCC) cell lines (Inamura et al, 2005; Romerio et al, 2000). In the present study, the effect of IFN α on ERK and MEK phosphorylation was not observed, although the phosphorylation level of both kinases was greatly increased in HeLa cells with extended culture time, which probably is because of the acquisition of serum growth factor stimuli. However, this p-ERK and p-MEK increase was not associated with IFN α stimulation.

Why different effects on ERK signaling by IFN α stimulation have been observed is unclear. One reason is probably that the function of the IFN-ERK axis in virus-infected cells and cancer cells is different. When cells require an immediate anti-virus function, with short-term IFN release, IFN α may activate ERK signaling,

while when cancer cells are exposed constitutively to IFN α , the ERK signaling may be inhibited. This may also suggest an IFN concentration-saturation threshold: when IFN concentration is saturating, it is more likely to exert a pro-apoptosis function thus ERK signaling is inhibited, while when IFN is at non-saturating concentration, it may conversely activate ERK signaling and thus promotes cell survival.

Cell type differences may also lead to the different observations. For example, some cells are sensitive to IFN α , or IFNs generally (IFN β , IFN γ or IFN α), whilst others have lost this response. If a cell is sensitive to IFN α -induced apoptosis, ERK signaling may be inhibited by IFN α , whereas ERK signaling may be enhanced or unaffected in IFN α resistant cells. Finally, it is necessary to avoid false positive observations, because ERK kinase activity (ERK phosphorylation) is easily influenced by experimental conditions including temperature, culture stress/pressure and serum growth factor stimuli.

In this study, why does IFN α have no effect on MAPK activation/inhibition in HeLa cells? Previously, one study has shown that MAPK activity can be induced by IFN α in T cells, but only in cells containing the Src-family kinase Lck (Lund et al, 1999). The expression level of Lck is not required for, and is not associated with, IFN α -stimulated STAT1 phosphorylation, activation and binding to DNA (Lund et al, 1999). These data indicate that Lck and active MAPK do not affect IFN α -induced growth arrest or induction of STAT1s DNA binding ability. Possibly in the HeLa cancer cell line, the Lck gene is less expressed or not expressed, thus IFN α did not activate ERK signaling, but could still activate STATs and induce ISGs expression, thus mediating some pro-apoptotic function.

5.3.4 IFN α stimulation decreased AKT signaling

In contrast to having no effect on ERK signaling, the data in this study showed that IFN α treatment inhibited AKT signaling and the downstream p70S6K1 kinase activity in HeLa cells. This is consistent with the recent report that AKT signaling was inhibited by IFN α in human cervical cancer progression (Ethiraj et

al, 2016). Given the fundamental importance of the AKT pathway in regulating cell proliferation and survival, this reduction in the activity of AKT may constitute a molecular mechanism for the anti-proliferative, pro-apoptosis activity of IFN α . Targeting the AKT pathway certainly strengthens the IFN-mediated anti-tumor effect. The mechanism of IFN α -inhibited AKT signaling probably is via IFN α -induced activation of protein phosphatase 2A (PP2A), a family of mammalian serine/threonine phosphatases that accounts for most of the regulation of serine/threonine kinase activity in cells (Janssens & Goris, 2001) and which directly dephosphorylates PI3K or AKT activating phosphorylation sites thus inhibiting AKT signaling (Trotman et al, 2006).

However, there are also some reports showing that IFN α can directly activate phosphatidylinositol 3-kinase (PI3K) (Uddin et al, 1997). The PI3K pathway has an important role in mediating gene transcription in response to both type I and type II IFNs and is essential for mediating their antiviral effects through the regulation of IFN-sensitive genes such as ISG15, IP-10 (also known as CXCL10) and IRF7 genes (Kaur et al, 2008a; Kaur et al, 2008b; Uddin et al, 1997). However, it is important to note that PI3K activation events reportedly occur very early (30 min) in the cells after stimulation with IFN α (Kaur et al, 2008b; Uddin et al, 1995). The biological significance of this effect remains unclear. In this current study, IFN α inhibiting AKT signaling was observed at a relative late stage, about 24 hours after IFN α treatment. A biochemical and functional characterization of the long-term events occurring upon treatment with IFN α is needed to better comprehend the mechanisms of action of this multifunctional cytokine.

5.3.5 PML-II regulation of ERK signaling

This study has, for the first time, demonstrated that ERK signaling was enhanced by depleting PML-II, which suggests an inhibition function of PML-II on this signaling pathway. The ERK pathway is strictly regulated by various factors at each step, for example by the expression of negative regulators such as Dual-specificity phosphatases (DUSPs) [also termed MAPK phosphatases (MKPs)]

(Jeffrey et al, 2007) and SPROUTY (SPRY) family member proteins (Cabrita & Christofori, 2008; Mason et al, 2006; Murphy et al, 2010). Particularly, DUSP5 (hVH3) acts as both a phosphatase and nuclear anchor for ERKs (Mandl et al, 2005) and DUSP6 (MKP-3) functions in cytoplasmic retention of dephosphorylated ERK2 (Karlsson et al, 2004). In most cases, DUSPs directly inactivate ERK by dephosphorylating threonine and tyrosine regulatory residues at the MAPKs catalytic loop (Caunt & Keyse, 2013). Therefore, if the expression of any of these proteins was dependent on PML-II, its depletion would impair the inhibitory effect on this pathway and result in ERK activation, as was observed. It would be interesting to carry out some experiments to investigate the effect of PML-II on the expression of these genes. Together, the impairment of these negative regulators' expression may contribute to ERK signaling enhancement. In conclusion, our results indicate that PML-II regulates the activity of the MEK/ERK pathway and consequently modulates cellular proliferation.

5.3.6 PML-II regulation of AKT signaling

In this study, knockdown of PML-II increased AKT activation, this is consistent with previous report that unspecified isoform(s) of PML inhibited AKT signaling (Trotman et al, 2006). Similar to the RAF/MEK/ERK pathway, the PI3K/AKT signaling pathway is also regulated by some feedback regulation mechanisms including PTEN (phosphatase and tensin homolog)-mediated negative regulation on PI3K/AKT pathway as PTEN deficiency closely correlates with AKT activation (Ito et al, 2009; Trotman et al, 2003). The expression of IRS1 (insulin receptor substrate 1) (Harrington et al, 2004) and RICTOR (rapamycin-insensitive companion of mTOR (mammalian target of rapamycin)) also positively regulates this pathway activation (Laugier et al, 2015; Sarbassov et al, 2005). PML-II may regulate the expression of these proteins, and thus affect AKT activation in HeLa cells. Phosphorylation plays an important role in the regulation of many diverse cellular processes and a delicate balance between protein kinases and protein phosphatases regulates these responses. Another possibility is that PML-II affects the activity of PP2A. Previous studies have demonstrated that PML binds to PP2A, and that loss of PML (in total, not a

specific isoform) impairs PP2A activity (Trotman et al, 2006), so knockdown of PML-II alone may also affect PP2A activity thus impairing the dephosphorylation of AKT and resulting in increased AKT activation. The idea that PML isoform II inhibits AKT and ERK is consistent with the PML protein's general function as a tumor suppressor.

5.3.7 Reason potential for IFN α less effectively inducing HeLa cell apoptosis

Although previous studies have implicated a variety of ISGs in mediating cell apoptosis, there are still many types of cancer cells that are less responsive to IFN α -induced apoptosis. In HeLa cells, IFN α increased the expression of several pro-apoptotic proteins, however IFN α only induced very limited amounts of apoptosis even at concentration reaching 10,000 U/ml, which is very high compared to pharmaceutical dose. We considered increasing the IFN α stimulation time to increase the cell death rate, however, it was impractical in this siRNA transient knockdown system, because after 48 hours siRNA transfection, the cells had already reached or were nearly confluent, making it impossible to continue another 3 days IFN α stimulation. Similarly, in human hepatocellular carcinoma cell lines, IFN α retarded G1/S transition and inhibited cell proliferation with no evidence of apoptosis (Inamura et al, 2005; Romerio et al, 2000). There are several factors that may contribute to HeLa cells' resistance to IFN α -induced apoptosis.

a. ISG induction is not strong and sustained

The expression of ISGs, including PUMA and TRAIL, may not be sufficient for inducing a large amount of cell apoptosis in HeLa cells. Because different cancer cells require different patterns of gene expression to achieve apoptosis, for example in melanomas, TRAIL and XAF1 are required for IFN α -induced cell apoptosis, and defects in induction of either TRAIL or XAF1 rendered cells resistant to apoptotic effects of IFNs (Chawla-Sarkar et al, 2001; Leaman et al, 2002), while in an ovarian carcinoma cell line, the induction of RIDs correlated with IFN-induced apoptosis (Morrison et al, 2001). In HeLa cell, there may be

some other unknown pro-apoptotic proteins which determine HeLa cells fate and that were not overexpressed in response to IFN α stimulation.

Based on the *in vitro* studies in melanomas and multiple myeloma (Crowder et al, 2005), strong and sustained induction of death receptor proteins such as TRAIL by IFNs has been reported as one means inducing cell apoptosis. In the work reported here in HeLa cells, IFN quickly increased the expression of these protein, but then the level decreased and was maintained at a relative low level rather than being sustained at high level. Probably this is one of the reasons why IFN could not induce a greater amount of cell apoptosis in HeLa cells.

This decrease in pro-apoptotic protein expression with longer induction may be due to a natural self-protection or self-renewal mechanism in cells. It is known that the interferon response is the first line of immune defense for cells to defeat virus infection. Rapid production of a large amount of cytokines is an essential mechanism for cells to defend against virus invasion. However, the continued production of cytokine would bring negative consequences for the cells. Therefore it is necessary for the cells to have a mechanism to re-regulate the expression of interferon or ISGs. The quickly decreased expression in specific cells such as HeLa cells may reduce the pro-apoptosis function and thus recover the resistance of the cell to IFN α -induced apoptosis.

- b. Other anti-apoptotic protein expression may also contribute cell resistance to IFN α induced apoptosis

The overexpression of inhibitors of apoptosis, such as XIAP (Liston et al, 2001), CIAP-1 or-2 (cellular IAPs) or FLIP (Flice inhibitory protein), has been implicated in resistance to apoptosis or chemotherapies in cancer (LaCasse et al, 1998; Schimmer & Dalili, 2005). For example, in melanoma cells, the expressed high levels of XIAP has been implicated in conferring resistance to TRAIL in melanoma cells. TRAIL functions in cell apoptosis are largely regulated by the expression of XIAP and cFLIP. Therefore, the final fate of cancer cells is dependent on the balance between anti-apoptotic and pro-apoptotic protein level.

- c. Cell sensitivity to IFN α may also determine IFN α efficacy

Cancer cell lines from different histologies respond variably to IFN α or IFN β or IFN γ induced apoptosis. For example, IFN β was more potent compared to IFN α 2 or IFN γ in inducing apoptosis in melanoma (Chawla-Sarkar et al, 2001), ovarian carcinoma (Morrison et al, 2001) and multiple myeloma cell lines (Chen et al, 2001), which correlated with stronger induction of TRAIL by IFN β . Thus melanoma cell lines, where both TRAIL and XAF1 were induced, were sensitive to IFN β induced apoptosis. To examine the relevance of differential activity of IFNs to HeLa cell apoptosis, it would be necessary to repeat these experiments using IFN β or poly(I:C), a synthetic analog of double- stranded RNA (dsRNA), stimulation in place of IFN α . Carrying out some experiments using a more IFN α -sensitive cancer cell line may also need to be considered.

d. ERK signaling pathway enhancement may also promote cell survival

The increased activation of ERK signaling by culture medium, probably through the growth factors contained in serum, may also be one of the reasons that cells showed resistance to IFN α -induced apoptosis. The enhanced pro-proliferation and pro-survival ERK signaling may also compensate for the inhibition on AKT signaling by IFN α . Together, the mechanisms that are responsible for a lack of response to IFN α are still not fully understood. Thus a better understanding of the mechanisms that underlie IFNs anti-tumor effect and the factors that are responsible for a lack of response to IFNs is desirable.

5.3.8 PML-II collaborates with IFN α to induce apoptosis

The function of PML in tumor suppression suggests that inactivation or downregulation of PML would provide an advantage for tumor development and progression. Indeed, the expression of PML protein is frequently downregulated in diverse types of human tumors and this downregulation often correlates with tumor progression (Gurrieri et al, 2004). In this study, depletion of PML-II increased cell survival in HeLa cells, a cervical cancer cell line. Mechanistically, depletion of PML-II results in downregulated expression of many anti-apoptotic proteins such as ISG15/54, OAS1, TRAIL and PUMA, through a mechanism of regulating the determinant transcription factor p53 or STAT1 expression and

recruitment at gene promoter. Moreover, the loss of PML-II increases pro-survival AKT and ERK signals and thus partly recovered ATK signal inhibited by IFN α and consequently increased cell resistance to IFNs-induced apoptosis. Taken together, PML-II positively regulates IFN α -induced pro-apoptotic protein expression and negatively regulates AKT and ERK signals activation thus provides a molecular basis for the involvement of PML-II in IFN α -mediated apoptosis in HeLa cells

Chapter 6 Final discussion

6.1 The unique C-terminal fragment of PML-II determines its distinct function in gene transcription

Multiple isoforms of tumour suppressor protein PML have been demonstrated to participate in various cellular activities (Bernardi & Pandolfi, 2007). The C-terminal of PML is thought to be associated with the specific biological properties of each isoform. In this study, the functional fragments of the C-terminal domain in PML-II was firstly investigated. The C-terminal fragments, particularly the regions covering 7b exon (645-758) defined by deletions $\Delta 1$ and $\Delta 2$, are essential for the PML-II regulation function in a type I IFN response, while the N-terminal RBCC structure is dispensable. The loss of the RBCC domains has no effect either on PML-II association with other transcription-related proteins or on PML-II binding at relevant promoters. However, the removal of C-terminal $\Delta 1$ and $\Delta 2$ sequences greatly impaired PML-II binding with transcription factors and consequently decreased gene transcription.

PML protein has been reported to interact with coactivator CBP via the N-terminal coil-coil motif that is found in all the main isoforms (Doucas et al, 1999). Consistently, both full-length PML-II and PML-V indeed interacted with CBP. However, the deletion of the N-terminal RBCC domain changed the binding characterization of both isoforms: PML-II- Δ RBCC could still interact with CBP while PML-V- Δ RBCC could not do so. Similarly, the full-length PML-II and PML-V were found to bind to chromatin remodelling complex subunit Brg-1, while PML-II- Δ RBCC and PML-V- Δ RBCC performed differently. While PML-II- Δ RBCC could form a complex with Brg-1, transcription factor STAT1 and coactivator CBP, suggesting an interaction can be mediated by the PML-II C-terminal fragment, PML-V- Δ RBCC failed to interact with Brg-1 or CBP.

Subsequently, a further testing was performed to determine the exact binding sites that are responsible for PML-II binding with transcription factor STAT1 and NF- κ B, coactivator CBP and chromatin remodelling subunit Brg-1. It can be

found that the key sequences for interaction with these proteins are at different sites in the C-terminal part of PML-II. STAT1 binding to PML-II was most affected by loss of residues 653-665, defined by mutations ΔB , ΔC and ΔD within $\Delta m1$, while NF- κB binding was mainly affected by mutation ΔD at residue 661 to 665, although NF- κB binding was also somewhat affected by ΔB or ΔC mutations. CBP binding was also preferentially affected by removing residues 661-665 of PML-II: this deletion sequence greatly disrupting PML-II binding with CBP. CBP binding ability to PML-II was also impaired by the $\Delta m1\Delta m2$, $\Delta 7$ and ΔB mutations to various degrees. Similarly, the ΔD mutation also disrupted Brg-1 binding with PML-II, because very little Brg-1 was precipitated from the cells transfected by ΔD mutant plasmid. It is interesting to note that the PML-II sequences required for Brg-1 binding are similar to those required for CBP and NF- κB binding. This suggests that one or more of these factors might be a direct interactor with PML-II whilst others interact indirectly via that primary interactor. It is also consistent with the idea that PML-II may function as a scaffold protein to recruit various transcription-related proteins and form a transcriptional complex.

It was recently reported that the unique C-terminal domain of PML-II can bind to PML-NBs independent of the shared N-terminal region (Geng et al, 2012). This is consistent with our observation and, taken together, it can be suggested that the C-terminal part of PML-II might replace full-length PML-II for some functions if it were to be expressed independently. In this context, we found that overexpressing PML-II deleted of its N-terminal RBCC domain further enhanced the expression of IFN β and ISGs while full-length PML-II only modestly increased the expression of these genes. This may suggest that removal of the RBCC domain actually gives a protein with enhanced activity in the expression of genes. The biological significance and mechanism of this effect remains to be determined, but it could reflect a greater functional availability of the PML-II C-terminal domain when less tightly tethered to PML-NB. Taken together, these results provided further evidence of PML-II, particularly its distinct C-terminal domain, playing a key role in the regulation of gene transcription.

6.2 PML functions in post-translational modification (phosphorylation and acetylation)

Many proteins that are resident in PML-NBs are subject to various post-translational modifications including SUMOylation (Fogal et al, 2000; Zhong et al, 2000a), ubiquitination, phosphorylation and acetylation (Cheng & Kao, 2012; Hofmann & Will, 2003). PML protein participates in diverse modifications to activate or suppress the function of these proteins. In this study, the knockdown of PML-II decreased transcription factor STAT1 phosphorylation in response to IFN stimulation, suggesting PML-II may facilitate IFN α -induced STAT1 activation. Similarly, overexpression of PML isoforms (I-VI) in human cells increased IFN γ -induced STAT1 phosphorylation, conversely, downregulation of PML by RNA interference was accompanied by a decrease in IFN γ -induced STAT1 phosphorylation (El Bougrini et al, 2011). Mechanistically, PML localization within the NBs and its SUMOylation at some/all of the specific lysines at 65, 160 and 490 are required for increased STAT1 phosphorylation in response to IFN γ stimulation, while the mutation of these lysines in PML-III abrogated its positive regulation of IFN γ -induced STAT1 phosphorylation (El Bougrini et al, 2011). Given the PML gene encodes PML-III from an mRNA that is very similar to PML-II mRNA, this may suggest PML-II SUMOylation is required for STAT1 phosphorylation in response to IFN α stimulation. In addition, the possibility cannot be excluded that PML-II directly regulates STAT1 upstream kinases JAK1 and TYK2. Further studies are required to determine the mechanism of PML-II regulating STAT1 phosphorylation. In fact, PML protein has previously been reported to regulate the activity of other kinases and activate transcription factors. For example, PML-IV promotes the phosphorylation of p53 by regulating homeodomain-interacting protein kinase-2 (HIPK2) kinase, which increases the rate of acetylation of p53 and consequently its transcriptional activation (Hofmann et al, 2002).

PML was also reported to modulate the activity of phosphatases. For example, the loss of PML delocalized PP1A and reduced its activity towards pRb, resulting in increased proliferation and reduced differentiation of neural progenitors (Regad et al, 2009). PML also positively regulates the activity of

phosphatase PP2A towards AKT in PML-NBs, and, as a result, losing PML leads to a more aggressive form of cancer (Trotman et al, 2006). In this study, knockdown of just PML-II alone also promoted AKT and ERK phosphorylation, and hence activation, in HeLa cells suggesting a negative regulation of PML-II on the activities of these kinases. This is probably because the loss of PML-II reduces the suppression/inhibition on AKT and ERK signals or on activation of the upstream kinase PI3K and CRAF/C-RAF, respectively. It could also be that depletion of PML-II disrupts AKT and ERK signaling pathway negative feedback loops that are mediated by DUSPs and PP2A phosphatase. Further studies are needed to elucidate the mechanism of PML-II negative regulation on ERK and AKT signals.

The function of PML-NBs/PML in the regulation of protein acetylation has been extensively demonstrated. For example, PML activates p53-dependent gene expression by promoting its acetylation by CBP in the PML-NBs (Pearson et al, 2000). In this study, the association of specific isoform PML-II with histone acetyltransferase CBP/p300 was demonstrated, and that the depletion of PML-II affects CBP/p300 recruitment at gene promoters (Chen et al, 2015). This suggested the possibility that PML-II positively regulates CBP-mediated acetylation of transcription factors or histones. However, PML was also reported to form complexes with multiple corepressors (c-Ski, N-CoR, and mSin3A) and histone deacetylase 1 (HDAC1), and to mediate tumour suppressor protein Mad transcriptional repression (Khan et al, 2001). This distinct function involving PML is most likely because of the individual functional differences among PML isoforms. Together, these data provide support for the idea that PML regulates protein acetylation by affecting acetyltransferase or deacetylase activity.

6.3 PML functions on histone methylation

Gene transcription activation is dynamic process which is accompanied by the presence or loss of some special histone modifications from the vicinity of the promoter and/or the gene. In this study, deletion of PML-II resulted in the enhancement of H3K9me3 at ISG promoters. This result is consistent with a recent study where silencing of PML enhanced the H3K9me3 level at the Oct4

gene promoter in P19 embryonal carcinoma (EC) cells (Chuang et al, 2011). Our study suggests that isoform PML-II may be one of the most important isoforms that participates in this process of methylated-histone enrichment/disposition, thus regulating gene transcription activation or repression. To further confirm this function, it will be necessary to test more repressive markers including HP1c, H3K27me3 and RIP140. It would be predicted that the knockdown of PML may enhance the recruitment of these repressive markers at IFN-responsive promoters. The H3K9me3 modification can be catalysed/mediated by multiple methyltransferases (HMTase) including SETD1 (or ESET), SUV39H1, SUV39H2, EHMT1(GLP) and EHMT2(G9A) (Kim & Kim, 2012). Particularly relevant may be the methyltransferases SETDB1 and SUV39H1, which reportedly interact with PML and regulate gene expression (Carbone et al, 2006; Cho et al, 2011). Therefore, it will be interesting to test whether loss of PML-II also affects the corecruitment of these HMTase to the promoters and thus their functions/activities.

A decrease of the supposed activating H3K4 trimethylation mark at promoters due to the depletion of Brg-1 or PML-II was not observed in this study, despite the reduction in their activity. The exact reasons for this remain unclear. However, recent studies showed that CBP/p300-mediated H3K27ac antagonizes with PRC2-mediated H3K27me2/3 in regulating polycomb target gene expression (Tie et al, 2009; Wang et al, 2010). Moreover, H3K4ac was found enriched at promoters of actively transcribed genes and located just upstream of H3K4me3, and the Set1-containing complex (COMPASS), which promotes H3K4me2 and -me3, also serves to limit the abundance of H3K4ac at gene promoters. This may suggest that the histone residue modifications H3K4ac and H3K4me2/3 are mutually exclusive (Guillemette et al, 2011). It is also reported that H3K4me3 enrichment/deposition at a promoter can be affected by other pre-existing histone modifications at this site, or by the duration of its residency (Howe et al, 2017). This suggests a possibility that the enrichment of H3K4me3 at promoter may be negatively affected by H3K4ac, the level of which was reduced at promoters by depleting either PML-II or Brg-1.

In order to further confirm the importance of PML-II for H3K4me3 enrichment/deposition, it would be interesting to test co-recruitment of H3K4me3 methyltransferases such as MLL1, MLL2 and SETD1 (Yokoyama et al, 2004; Zhang et al, 2015). Given that the recruitment of these methyltransferases requires specific transcription factors and coactivators (Katada & Sassone-Corsi, 2010; Narayanan et al, 2007; Okuda et al, 2014), it therefore can be postulated that the recruitment of methyltransferases to target gene promoters is most likely affected by depleting PML-II or Brg-1. To extend study of the function of PML-II positive regulation on gene transcription, it would be better to test the effect of PML-II on the recruitment of more activating markers, such as AcH3 and RNA Pol II. Knockdown of PML-II should drastically reduce AcH3 and RNA Pol II recruitment at a gene promoter.

PML protein can physically associate with many histone modifying enzymes and enzymatic components of chromatin remodeling complexes, such as protein acetyltransferase CBP/(p300), deacetylase (HDAC1/2/3/7, SIRT1 and SIRT5) (Guan et al, 2014; Morey et al, 2008; Wu et al, 2001), methyltransferases (SETDB1 and SUV39H1) (Carbone et al, 2006; Cho et al, 2011), component of polycomb repressive complex, EZH2 (Villa et al, 2007), and epigenetic regulator UHRF1 (Guan et al, 2013). This suggests that investigating the role of PML in epigenetics and chromatin organization will be a key direction for future study. The function of PML-II in gene transcription makes it one of the ideal isoforms on which to conduct these interesting studies.

6.4 PML-II affects chromatin remodeling SWI/SNF complex

As one of two possible core catalytic subunits of SWI/SNF complex, Brg-1 plays an important role in transcription. Brg-1 has been reported directly to regulate the expression of IFN β and also multiple ISGs (Agalioti et al, 2000; Cui et al, 2004; Huang et al, 2002; Pattenden et al, 2002). Similarly, in this study the expression of IFN β and many ISGs was also decreased by depleting Brg-1. Importantly, this decrease was further enhanced by double knockdown of PML-II and Brg-1, this may suggest PML-II collaborates with Brg-1 to regulate gene transcription.

Several models of SWI/SNF complex (Brg-1) binding to chromatin have been proposed. The data in this study supported the idea that Brg-1 recruitment to its target required gene specific transcription factors, because as has been observed, over a time course, transcription factor STAT1 was first activated and recruited to a gene promoter after stimulation and that this occurred significantly earlier than the recruitment of Brg-1 and BAF155, and some other transcription coactivators.

Similar to Brg-1, BRM also plays an important role in the expression of some ISGs (Torti, 2012). Although the functions of Brg-1 and BRM are partly similar or redundant, BRM and Brg-1 complexes regulate different gene expression profiles. In this study, PML-II has a stronger effect on Brg-1 and BAF155 binding at promoters of some genes such as ISG54 and IFITM3 (named as an early gene with the IFN response), while it has a relatively smaller effect on genes like ISG56 and GBP1, although the expression of all of these gene was similarly downregulated by depletion of PML-II. This may suggest the expression of some ISGs is Brg-1-dependent, while expression of others is not. Considering the previous expression characterization of these genes, it seems the expression of some early response ISGs is more likely regulated by Brg-1 and/or BRM (maybe in combination), while the expression of later response genes is less dependent on Brg-1 (and may depend on BRM alone). Also, it is possible that a switch occurs between Brg-1 and BRM during the process of gene transcription, namely Brg-1 participates in transcription initiation first, while BRM plays a role at later stage. Therefore it would be interesting to identify genes that are more BRM dependent by knockdown of BRM and to test the dynamic recruitment/binding pattern of BRM at different gene promoters.

In this study, PML-II bound to Brg-1, CBP and other transcription factors to form a transcription complex (Figure 4.2.7 and 4.2.8), and the depletion of PML-II affected the binding of such transcription-related proteins at promoters. In addition, the depletion of PML-II down-regulated the expression of the SWI/SNF ATPase subunit BRM as well as core subunit BAF47, down-regulation of which influences Brg-1 and BRM stability. Accordingly, it can be concluded that the regulation by PML-II of chromatin remodeling SWI/SNF complex

activity occurs via two distinct methods, including decreasing subunit protein expression (BRM and BAF47), and regulating subunit protein (Brg-1 and BAF155) binding/recruitment at the promoter.

Besides SWI/SNF, there are at least four families of chromatin remodelers in eukaryotes such as ISWI, NuRD/Mi-2/CHD, INO80 and SWR1 (Wang et al, 2007). Each remodeler complex has unique protein domains (helicase, bromodomain) in their catalytic ATPase region and also has different recruited subunits; their functional characteristics thus are different. The ISWI-family remodelers have been shown to play central roles in chromatin assembly after DNA replication and in the maintenance of higher-order chromatin structures, which is completely different to the role of SWI/SNF remodelers in disordering nucleosomes. NuRD/Mi-2/CHD remodeling complexes primarily mediate transcriptional repression in the nucleus and are required for the maintenance of pluripotency of embryonic stem cells (Wang et al, 2007). SWI/SNF and ISWI remodelers are very well studied so far. To further expand the study of PML-II transcriptional regulation function on chromatin remodeling, it may be necessary to test the effect of PML-II depletion on ISWI or NuRD/Mi-2/CHD remodeling complexes activity.

6.5 Proposed model of gene transcription and PML-II functions in this process

In this study, the dynamic changes in the binding of STAT1, CBP, PML-II, Brg-1 and BAF155, and the enrichment/deposition of H3K4me3 and H3K9me3, at gene promoter in response to IFN α stimulation were tested (Figure 6.1). The binding of STAT1 at a promoter was first to reach the highest level and this then decreased, which was closely associated in time with significant new mRNA expression, however the highest level of gene expression was 1-2 hours later than that of STAT1 recruitment at the promoter. Similar to STAT1, the level of H3K4me3 modification at ISG promoters was also greatly increased by IFN α induction. Conversely, the level of H3K9me3 at promoters was immediately decreased by IFN α . The timing of CBP binding at the promoter was very similar to that of H3K4me3. The binding of PML-II, BAF155 and Brg-1 at ISG

promoters was also increased by IFN α stimulation, and the maximum level of PML-II and BAF155 present at a promoter was at 8 h after stimulation, while the highest enrichment level of Brg-1 at a promoter was at 6 h after stimulation which is about 2 hours earlier than PML-II and BAF155 binding. The time difference of Brg-1 and BAF155 recruitment at a promoter is consistent with the previous observations that the recruitment/assembly of components of chromatin remodeling complexes was sequentially within condensed chromatin, Brg-1 having earlier recruitment kinetics than BAF155 (Memedula & Belmont, 2003; Ryne et al, 2009). In order to acquire the complete picture of gene transcription initiation dynamics, it will be necessary to test other transcription-related mechanisms including the recruitment of RNA pol II, the general transcription machinery and the enrichment/disposition of histone modification (acetylation).

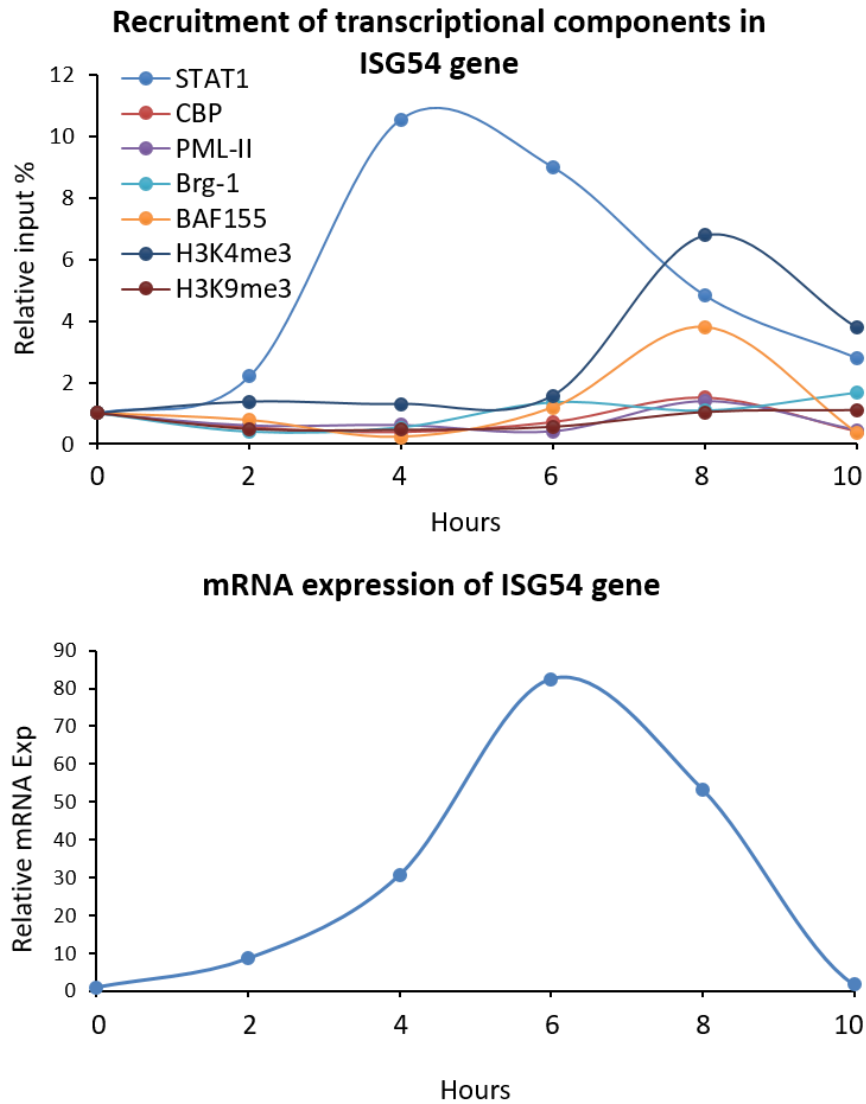


Figure 6.1 Upper: The recruitment pattern of transcriptional components in ISG54 gene during induction by IFN α . Lower: Expression of ISG54 mRNA following IFN α induction.

Altogether, based on the DNA binding time course of various proteins to ISG promoters and the dynamic changes to histone modifications in those promoters, a model of gene transcription at ISGs and the regulation by PML-II on the process was proposed. Firstly, gene transcription is initiated by transcription factor STAT1 phosphorylation (within 1h), meanwhile the level of H3K9me3 at promoter is decreased immediately (probably earlier than the time point tested at 2h after stimulation). Then STAT1 moves into nucleus and binds to promoter DNA (4-6h), followed by chromatin remodeling core enzymatic subunit Brg-

1/BRM enrichment (6-8h), and scaffold protein PML-II, and co-activator CBP and SWI/SNF scaffold protein BAF155 (8h) recruitment and by 8h, when H3K4me3 enrichment is observed, the whole transcription complex is assembled at the promoter. Meanwhile, mRNA transcription reaches the highest level (6-8h). This is very consistent with the generally accepted concept that induction of gene transcription involves sequential/ordered recruitment of various activators, coactivators, regulators/mediators and the general transcription machinery.

Based on the data in this study, it can be concluded that PML-II regulates SWI/SNF complex functions in gene transcription. PML-II is required for forming and stabilizing the whole transcriptional complex which contains various factors including transcription factors (STAT1), coactivator (CBP), and chromatin remodeling complex (Brg-1 and BAF155) (Figure 6.2). Depletion of PML-II impaired transcriptional complex stability and component protein binding at a promoter. According to this model, PML-II mainly functions as a scaffold protein, providing stability/integrity for the complex.

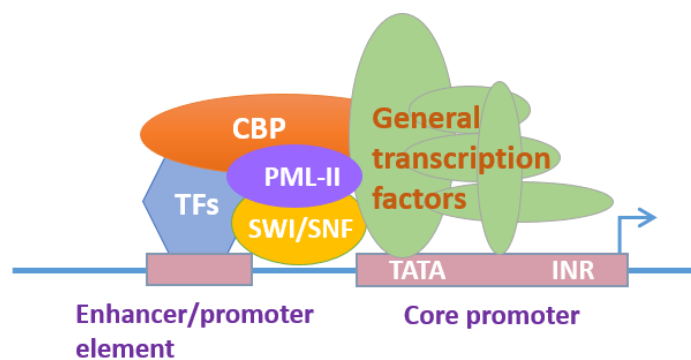


Figure 6.2 A model of PML-II function as a scaffold protein in gene transcription

However it is also possible PML-II may function as modifier/mediator through its association (direct or indirect) with SWI/SNF complex and transcriptional components by regulating their PTMs, hence facilitating their recruitment and assembly at promoters. Principal among the PTMs known to be positively

regulated by PML are phosphorylation and sumoylation. The loss of PML-II would impair the PTMs of these factors and transcriptional complex assembly and gene transcription (Figure 6.3). Collectively, this study establishes the relationship between PML-II with SWI/SNF chromatin remodeling complex, and provides evidence of PML-II's role in regulation of chromatin remodeling and histone modification, suggesting PML-II as a master regulator in gene transcription.

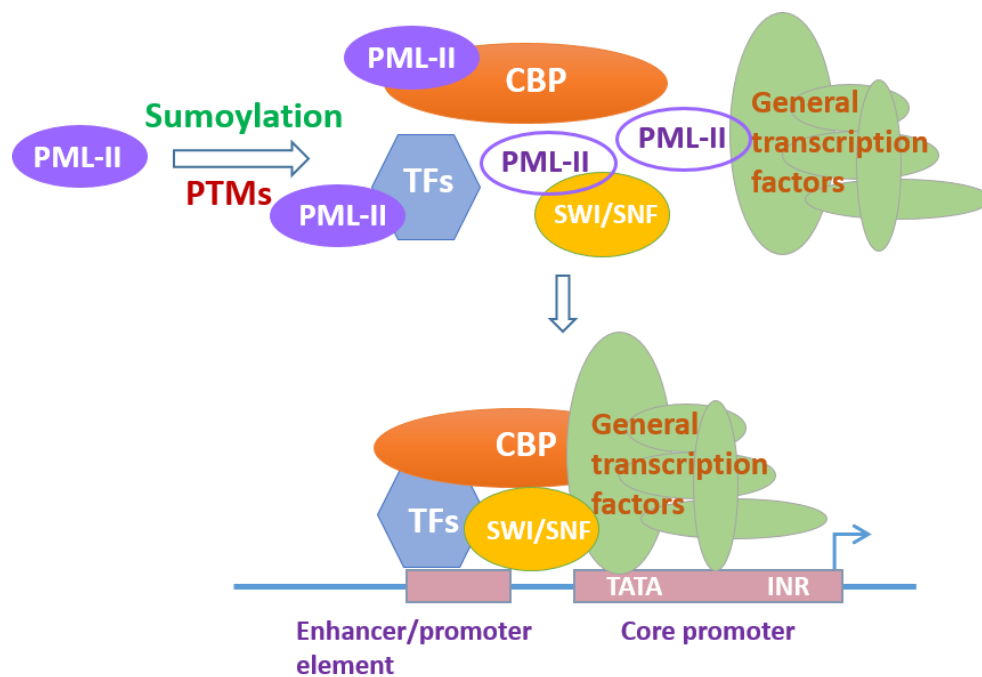


Figure 6.3 A model of PML-II functions as modifier of factors involved in gene transcription

6.6 PML functions in tumour suppression

A number of cellular activities of PML protein are related to anti-tumour activity. For example, PML activates p53 and recruits it into the PML-NBs where PML binds to and inhibits the negative regulator MDM2 by sequestering MDM2 to the nucleolus thus enhances p53 stability and mediates p53 pro-apoptotic function (Bernardi & Pandolfi, 2003; Takahashi et al, 2004). In this study, the PML-II isoform was found to have an effect of regulating p53 expression and the expression of a pro-apoptotic p53-dependent gene, PUMA, in response to IFN α

stimulation. These results suggested that PML-II is an essential isoform to regulate p53-mediated pro-apoptosis function.

Overexpression of PML in gastric cancer cells recently was reported to significantly increase cell apoptosis, resulting in decreases in cell growth; this effect of PML appeared to be conducted through p73-mediated modulation of apoptosis-associated genes, Bcl-2, Bak and caspase-9 (Xu et al, 2015). PML also negatively regulates the AKT-mTOR pathway, so inhibiting cancer cells angiogenesis (Bernardi et al, 2006). PML was also found to exert proapoptotic activity through the regulation of a large complex involving PP2a, AKT, and the inositol triphosphate receptor (IP3R), which ultimately controls calcium flux to the mitochondria (Giorgi et al, 2010).

Similar to previous observation with total PML, PML-II in this study negatively regulated AKT activity as its depletion increased the phosphorylation level of AKT and the downstream kinase p70S6K1. Interestingly, PML-II also demonstrated a negative regulation function on ERK signaling and the expression of ERK-dependent genes, c-Fos and c-Myc. PML was reported to regulate AKT activity by recruiting PP2a to PML-NBs, thereby dephosphorylating and inactivating AKT (Trotman et al, 2006). It is not sure whether PML-II regulates AKT signaling through the regulation on PP2a or by another means. Further study is required to investigate this remaining question. Collectively, the data do indicate that PML-II negatively regulates ERK and AKT signaling pathways, suggesting an important role for PML-II in the inhibition of cancer cell proliferation and in promoting cell apoptosis. The data also provide a molecular basis for the PML tumour suppression function, and suggest that this function resides, in part at least, in the PML-II isoform specifically.

6.7 PML regulates IFN-induced apoptosis

The products of a group of ISGs are thought to be the primary effectors that mediate the IFN-anti-tumour function. For example, in myeloma and

hepatocellular carcinoma cells, the growth inhibition and pro-apoptosis function induced by IFN α is mainly dependent on PML and TRAIL induction (Crowder et al, 2005; Herzer et al, 2009). Moreover, the expression level of PUMA, a p53-dependent gene, was also increased in response to IFN α stimulation in human myeloma cells suggesting the possibility that PUMA is related to IFN α -induced apoptosis (Gómez-Benito et al, 2007).

Similarly, in this study, IFN α stimulation greatly induced the expression of pro-apoptotic proteins including ISG15, ISG54, OAS1 and TRAIL and PUMA in HeLa cells. In addition, IFN α treatment also inhibited AKT signal activation which will likely contribute to IFN α -induced apoptosis. IFN-induced cell apoptosis is complex: the eventually cell death may be a result of collaboration of multiple IFN α -regulated mechanisms. This study has shown IFN α not only increases antiapoptotic protein expression but also inhibits pro-survival AKT signaling. This suggests the function of IFN α to promote cancer cell apoptosis is most likely a cumulative effect of expressing many pro-apoptotic proteins and the inhibition of multiple pro-survival signaling pathways.

In this study, depletion of PML-II reduced the death of cells in response to IFN α stimulation. This provides further support to the idea that PML is required for the efficient induction of apoptosis by IFNs (Quignon et al, 1998; Wang et al, 1998) and also suggests that PML-II is one of the important isoforms involved in IFN α -mediated apoptosis. Mechanistically, knockdown of PML-II greatly decreased the expression of genes including TRAIL, which is consistent with a previous observation that loss of all PML decreased TRAIL expression in hepatocellular carcinoma cells (Crowder et al, 2005). Also, the expression of many pro-apoptotic ISG proteins and PUMA was also decreased by PML-II depletion. This is consistent with a previous observation that the growth inhibition effects of IFN α correlated with the presence of PML in myeloma cells (Crowder et al, 2005). IFN α -induced apoptosis in hepatocellular carcinoma also involves PML (Herzer et al, 2009). Taken together, PML-II regulation of IFN α -induced pro-apoptotic protein expression, and inhibition of pro-survival AKT and ERK signaling pathways provides a molecular basis for the involvement of PML-II in IFN α -mediated apoptosis in HeLa cells.

6.8 Loss of PML-II may contribute cell resistance to IFN-mediated apoptosis

Type I IFNs have been used for the treatment of several types of haematological malignancies and solid tumours (Ferrantini et al, 2007; Rizza et al, 2010). However, there are many type of cancer cells that are less responsive to IFN α -induced apoptosis. Clinically, a substantial proportion of patients fail to respond to IFN treatment, although beneficial effects of IFNs in some malignant disease patients was observed. In HeLa cells, IFN α induced a very limited amount of apoptosis although IFN α considerably/significantly induced the expression of a group of pro-apoptotic proteins including ISG15/54, OAS1, TRAIL and PUMA. The factors that may contribute to HeLa cells resistance to IFN α -induced apoptosis have been discussed in Chapter 5. Here the focus will be on the resistance probably due to the loss of PML/PML-II protein.

The expression of PML tumour suppressor protein is frequently downregulated in diverse types of human tumour and this downregulation often correlates with tumour progression (Gurrieri et al, 2004; Trotman et al, 2006). In a mouse model, loss of the *Pml* gene markedly accelerates tumour onset, incidence and progression (Trotman et al, 2006). Cells from PML-deficient mice show severe apoptotic defects, including a strongly decreased sensitivity to death receptor-mediated apoptosis (Wang et al, 1998).

Our and others' previous studies have confirmed that PML protein is required for IFN-induced apoptosis. It therefore can be proposed that the basis of resistance to IFN α -induced apoptosis in HeLa cells and other resistant tumour cells is that the protein level of PML-II is lower compared to IFN α -sensitive cancer cells, although the mRNA level may be still detectable. Full length PML-II is very hard to detect in HeLa cells and in other tumour cell lines (Wright, PhD thesis, University of Warwick, 2010). A reduced amount of functional or active PML-II will affect IFN α -induced pro-apoptotic protein expression and thus should

increase cell resistance to IFN α -induced apoptosis. The inactivation or downregulation of PML would provide an advantage for tumour development and progression.

The low level of PML-II may arise by the protein undergoing post-translation modification which eventually results in PML-II degradation via the ubiquitination-proteasome pathway. Alternatively, its expression might be affected by changes in alternative RNA splicing patterns. Collectively, the downregulation or inactivation of PML-II may be a critical factor leading to cancer cells becoming less responsiveness to IFN α -mediated apoptosis. Thus a better understanding of how PML protein (particular PML-II) is involved in the response to IFNs may ultimately lead to better utilization of IFNs in the treatment of cancer.

6.9 Preventing PML-II degradation sensitizes IFN α -induced apoptosis

To overcome the possible resistance of cancer cells to IFN α -induced apoptosis, many strategies have been reported which include inhibiting the expression of natural inhibitors of apoptosis, for example targeting XIAP to sensitize cancer cells to IFN-mediated apoptosis (LaCasse et al, 1998; Leaman et al, 2002), or overexpression of XAF-1 (X-linked inhibitor of apoptosis-associated factor-1) to sensitize melanoma cells to TRAIL-induced apoptosis by modulating cleavage of XIAP (Leaman et al, 2002). Also, the concept of combination therapy including IFN has been accepted, for example, the combination of IFN α and imatinib (an inhibitor of Bcr-Abl tyrosine-kinase) significantly increased the synergistic/additive effects to inhibit chronic myeloid leukemia (CML) cell growth compared to single treatment (Kano et al, 2001; Simonsson et al, 2011; Talpaz et al, 2013). Pre-treating TRAIL-resistant melanoma cells with IFN β for 12–24 h also increased the sensitivity of cells to TRAIL-induced apoptosis and cleavage of XIAP (Chawla-Sarkar et al, 2001; Kumar-Sinha et al, 2002).

In view of the important role of PML/PML-II in the regulation of IFN α -mediated apoptosis, the partial or complete loss of PML/PML-II protein certainly affects

the efficacy of IFN α -mediated apoptosis. Recently, it was reported that overexpression of PML in gastric cancer cells significantly increased cell apoptosis (Xu et al, 2015). Therefore it can be proposed that the overexpression of PML/PML-II/PML-II- Δ RBCC protein may be a possible approach to improve PML tumour suppression function and/or to increase the sensitivity of cells to IFN α / β -induced apoptosis.

In recent years, targeting the PML ubiquitination pathway, thus preventing its degradation, has become an attractive approach for anti-cancer therapy. Aberrant PML protein degradation has been observed in human cancers from multiple origins including prostate adenocarcinoma, colon adenocarcinoma, lung carcinoma, breast carcinoma, lymphoma, central nervous system (CNS) tumours and germ cell tumours (Chen et al, 2012). Proteasome-dependent degradation is proposed to be a mechanism by which tumour cells restrict the expression of PML protein. Since ubiquitination is the major posttranslational modification that controls protein degradation through the proteasome, targeting the PML ubiquitination pathway therefore becomes an attractive strategy for anti-cancer therapy.

The approaches that can be utilized to prevent PML degradation include ubiquitin E3 ligase inhibitors, inhibitors that disrupt the interaction of PML with its E3 ligases, or targeting PML phosphorylation and SUMOylation by introducing kinase inhibitors, because both these posttranslational modifications are often required for the subsequent ubiquitination events, e.g. the action of SUMO-dependent ubiquitin ligases. Accordingly, targeting ubiquitination mechanisms to prevent PML degradation may provide an opportunity to recover PML tumour suppressive functions and to improve the activity of various death receptors, IFN α -induced or other drug-induced cell apoptosis. The data in this study have shown that PML-II is one of the important isoforms that mediate IFN α -induced anti-tumour activity and thus preventing PML-II degradation may improve cell sensitive to IFN α -induced apoptosis.

6.10 Model of PML-II regulation of IFN α -mediated apoptosis

According to the results in this study, a model of PML-II tumour suppressor function was proposed (Figure 6.4). According to this model, IFN α stimulation increases the expression of PML-II which in turn positively regulates IFN α -induced pro-apoptotic protein expression, contributing to IFN α -mediated anti-tumour activity. The intrinsic inhibitory effect of PML-II on AKT and ERK signaling also contributes to IFN α exerting this function. This study thus provides a mechanism to explain IFN α induced cancer cell apoptosis and the important regulatory role of PML-II in this biological process. This study may also suggest a novel strategy in cancer therapy by combining IFN α with PML/PML-II protein expression/accumulation. Taken together, understanding the mechanisms by which PML-II functions in these pathways is important for our comprehension of the proper function of PML/PML-II and possibly to develop novel targets to improve IFNs-mediated anti-cancer activity.

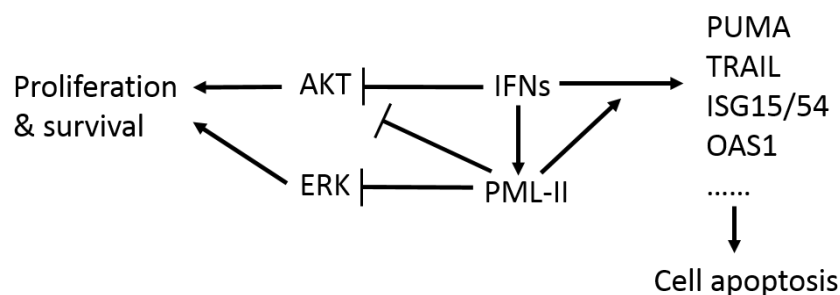


Figure 6.4 A model of PML-II functions in IFN α -mediated apoptosis.

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